University College London

Nicola D'Arcy

Exploring the Nature and Diversity of Microorganisms in Healthcare and Educational Settings

Eastman Dental Institute

Doctor of Philosophy

Supervisors: Dr David A. Spratt and Professor Nigel Klein

2014

I, Nicola D'Arcy confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed…….

Date……

Abstract

Many human populations spend approximately 90 % of their time indoors, yet relatively little is known about the microbial communities associated with indoor environments. This is despite knowledge that these microorganisms can contribute to adverse health effects, including the acquisition of healthcare-associated infections, which cause significant morbidity and mortality. The concept of the 'indoor microbiome' is relatively new and to date, few studies have been field-based, systematic and long-term. Hospitals in particular, are unique environments which have been shown to drive microbial evolutionary processes as they contain a different sub-set of the human population. The study of the hospital microbiome could have important implications for healthcare and infection control.

This thesis explores a range of methods for investigating microorganisms in different indoor environments, including a classroom and outpatient's waiting areas and wards in a hospital. Results show that the classroom is much more heavily contaminated in terms of total viable counts (TVCs) of bacteria recovered than the hospital environment. This was thought to be attributed to the absence of a strict cleaning regime in the classroom. High-touch items were less contaminated than other objects, likely due to them being obvious cleaning targets. Potential pathogens, including a number of Enterobacteriaceae were cultured from the classroom, outpatient's waiting area and ward. Virus nucleic acid was recovered from an

outpatient's area, including norovirus and rotavirus RNA. Adenovirus DNA was frequently isolated throughout a 3 month screening protocol and there appeared to be evidence to suggest that a viral marker may be more appropriate than TVCs for identifying viral contamination. Human-associated bacteria were found to be dominant on a hospital ward over a 12 month longitudinal screening study and the presence of numerous bacterial taxa, which may be of concern in the context of paediatrics and immunodeficient patients, was also demonstrated.

Acknowledgements

I would first like to thank my supervisors Dave Spratt and Nigel Klein for taking on my project and for their encouragement and support. Both were exceptionally calming during the stressful times.

Thanks to Louise, Melisa, Paula and Jon at HIRC for helping to kick-start the project Lena for proof-reading and Dimitrios Margaritis for the maps of sampling locations displayed throughout this thesis. I would also like to thank Anna Tymon and those at the Eastman Dental Institute for their help. Thanks to all at the Camelia Botnar labs, especially Elaine Cloutman-Green for lab space, countless cups of tea, and advice. Thanks also to Kathryn Harris, John Hartley and Garth Dixon for providing practical help, reagents and knowledge and to Paul Locke and his team for their wisdom and support. Ronan Doyle provided barcode primers used in Chapter 6 and thanks also to him for bioinformatics support. Thanks to Infection Control for access to the wards and data and to all on Tiger and Koala who put up with me getting in the way taking my swabs; particularly Tom Kennedy.

Samana, thanks for the advert for the PhD! Thanks also to her and the rest of my amazing friends; Taemi, Paul, Gron, Alex, Kylie, Chris, Kara, Quinton, Paul and Kate. Without their support and that of others too many to mention, I wouldn't have been able to focus on the task at hand and complete the project. Thanks to Chris for being so wonderful and supportive.

Finally, I would like to thank my family, Mum, Will, Bob and my Grandparents. Without their encouragement, love and endless support, I would not have achieved anything in life. I hope you’re proud.

Table of Contents

List of Figures

List of Tables

1. Introduction

1.1 Overview

Microorganisms such as bacteria and viruses have evolved with primates over millions of years and as humans have moved into indoor environments, so too have various forms of microbial life. People have long been aware of microorganisms in their surroundings and it is thought that exposure to some species may even aid the development of the immune system and provide protection from conditions such as asthma (1), (2). Despite a large proportion of human populations spending approximately 90 % of their time indoors (3), relatively little is known about the microbial composition of these indoor environments. Whilst research has been conducted in specific indoor locations for specific microorganisms, information regarding total microbial populations in human environments is lacking (4). This is particularly true of viruses and airborne microorganisms as they can be difficult to isolate and quantify (5). The Earth plays host to more than 10^{30} microbial cells (6) but despite this abundance, knowledge and understanding of the microbial ecology of different environmental niches has not yet been fully explored.

The buildings in which people live, work and socialise and their microbial inhabitants are known to have an impact on health. Sick building syndrome (7), exposure to moulds from flooding and damp (8), the role of ventilation (9), (10) and the role of the environment in the spread of nosocomial infection (11), (12), (13) are well documented. In spite of this knowledge, these health issues are still of concern and adverse effects linked to indoor spaces continue to be reported.

The concept of the 'indoor microbiome', the ecological community of microorganisms within a given space, is relatively new and despite the availability of published articles, few studies which analyse the microbial diversity within indoor environments have been field-based, systematic and long-term. Currently, there is also a lack of standardisation of sampling and analysis methods, meaning that data are hard to compare across research groups (14). Therefore, there are a number of stand-alone studies that cannot provide practical applications for the research. Few studies combine air and surface sampling and little is known about whether environmental microbial quantity and composition relates to risk of infection. Methodologies to investigate the indoor microbiome have been traditionally centred on culture-based techniques but these have a tendency to underestimate the complexity of microbial diversity, as it is widely recognised that only 0.1 – 10 % of bacteria are cultivable (15), (16), (17). Research that isolates viruses from indoor locations is beginning to appear in the literature (18–21) but the methods are not fully developed, so findings tend to be general or inconclusive. Problems arise due

to the fact that sampling can damage virions (22), the isolation of live virus is laborious and costly and some species have no *in vitro* growth model.

Nucleic-acid based methods are routinely used in clinical diagnostics, with a number of assays available for the simultaneous detection of multiple pathogens but these have yet to be validated for use with environmental samples. With the increase in use and popularity of non-culture based analysis methods for determining microbial diversity, the concept of the indoor environmental microbiome is being introduced.

Investigating the indoor environment and its associated microorganisms could allow a greater understanding of the means of transmission of some pathogens. It could also provide information as to the effectiveness of measures designed to reduce infection such as cleaning, ventilation, building design, or choice of furnishings. Without long-term, in-depth studies to quantify and identify microbial diversity, systematic, informed measures to reduce risk cannot be taken. Effective surveillance of environmental microorganisms can have a significant benefit to health. For example, cases of hospital-acquired pneumonia in Germany due to air-associated *Legionella pneumophila* were reduced from 16.6 % to 0.1 % over the course of a 6 year sampling and monitoring programme (23).

There is a need for investigations that are concerned with understanding what is present, where, how this changes with regards to temporal factors and importantly, why a space may have a particular microbial profile and what that means in terms of

human health. Factors such as increasing drug resistance for example, can mean that certain species of bacteria which were once not considered to be of great concern, become a health-risk (24). In particular environments, species that are non-pathogenic to the majority of the population can become a risk, for example to the long-term hospitalised (25) or the immunocompromised (26). It is therefore important not just to investigate the presence of specific pathogenic taxa. The community context of these bacteria must also be understood because the abundance of a given pathogen is likely to be a function of the other taxa it interacts with within its ecological niche.

Understanding the causes and consequences of the microbial inhabitants of indoor spaces is not only important and interesting from an ecological point of view but is vital to reduce or prevent the spread of disease.

1.2 The indoor microbiome: current research

The concept of ‘indoor ecology’ as a collaborative effort to understand the microbial composition of our environments is relatively new. As previously mentioned, culture-based techniques vastly underestimate the diversity of microorganisms present but with the advent of molecular techniques, this limitation in our ability to discover novel species and gain a better insight into microbial ecosystems has been lifted. This has become even more apparent with the recent development and use of next-generation sequencing (NGS) which allows the sequencing of vast numbers of microorganisms from the same sample, at the same time in a much shorter time

period than traditional sequencing. This technology (discussed further in Chapter 6) has led to the initiation of various microbiome projects with a view to understanding the microbial communities within us and around us. The Human Microbiome Project (HMP), based at the National Institutes of Health, has conducted extensive research into the microbial ecology of humans (27). The project has produced microbial maps of the human body and aims to discover how changes in the microbiome are associated with health and disease. Over 190 scientific articles have been published to date by its collaborators since the project's launch in 2008. Major findings so far include the discovery of a link between oral and gut microbiota and atherosclerosis and cardiovascular disease (28).

More recently, the Earth Microbiome Project (EMP) has begun with the aim:

"*...to attempt to characterize the global microbial taxonomic and functional diversity for the benefit of the planet and mankind*" (29).

The EMP is aimed at examining global microbial communities using metagenomics: the study of genetic material directly extracted from environmental samples, metatranscriptomics: the study of transcribed genetic regions directly from environmental samples and sequencing to produce a 'Global Gene Atlas'- a vast database of microbial communities. It is proposed that this database will enable advances in our understanding of the ecology and evolution of previously unknown species. The EMP is predominantly concerned with natural samples such as soil,

water and outdoor air and does not currently place a great emphasis on indoor environments. However, the Hospital Microbiome Project, as part of the EMP aims to conduct large-scale environmental screening of a hospital in the United States (US) with a view to investigate microbial communities. The availability of scientific literature spanning the last few decades regarding the indoor microbiota indicates that this is an area of interest to the scientific community. However, research focussed on the analysis of indoor microbial communities has, to date, been conducted seemingly haphazardly with no defined standard methodologies or data analysis protocols.

Much of the prior research conducted has been entirely culture-based and has had a tendency to focus on the isolation of specific pathogens (30), (31), (32), (33). The Microbiology of the Built Environment network (www.microBE.net) is one of the only groups attempting to bring together research conducted in this area and encouraging collaborative work. In the United Kingdom (UK), research in this field is currently very limited with only a few laboratories being involved and none (at the time of writing) involved in using NGS technologies to characterise building microbiology.

Table 1.1 shows a very small selection of work reporting bacterial counts and basic taxonomic analysis from air and surface samples collected from indoor sources, not using NGS.

Table 1.1: A selection of work reporting bacteria counts and basic taxonomic analysis from air and surface samples collected from indoor sources prior to the availability of next-generation sequencing

Author	Location	Method	Culture Medium	Average CFU	Predominant genera or groups
Jo (31)	Bar School Home	Andersen air sampler	TSA[a]	3890 / m^3 1002 / m^3 2512 / m^3	Not reported
Newman (34)	ITU[b]	Swabs, settle plates	Blood agar, MacConkey	10-100	CNS[c], *Bacillus*
Obbard (35)	Hospital	Andersen air sampler	TSA	890 / m^3 (lobby) 325 / m^3 (ward)	CNS, *Corynebacterium, Acinetobacter*
Bartlett (36)	School	Andersen air sampler	TSA	325 / m^3	*Micrococcus, Staphylococcus*
Seino (37)	Underground concourse	Impactor air sampler	Plate count agar	150 - 1380 / m^3	Gram positive cocci
Augustowska (38)	Pneumonia ward	Slit air sampler	Blood agar	257.1 – 436.3 / m^3	Gram positive cocci
Kim (39)	Hospital Kindergarten Nursing home	Andersen air sampler	Not reported	404 / m^3 931 / m^3 294 / m^3	*Staphylococcus, Micrococcus, Corynebacterium, Bacillus*
Ferroni (40)	Cystic fibrosis ward	Omega air sampler	TSA	583.4 / m^3	Not reported

Continued over

Author	Location	Method	Culture Medium	Average CFU	Predominant genera or groups
Al-Shahwani (41)	Hospital	Not reported	Not reported	478.6	Not reported
Okten (42)	Paediatric Unit	Settle plates	BHI[d] with 5% blood	43-234 / plate	*Staphylococcus, Bacillus, Corynebacterium*
Rettberg (43)	Spacecraft assembly facility	Airport MD8 Cotton swabs	Gelatine filter BHI	1 – 85 / m^3 > 3000 / swab	*Bacillus, Micrococcus, Staphylococcus, Acinetobacter*
Wojgani (44)	Hospital door handles	Contact plates	TSA	> 300 / plate	Not reported
Gaudart (45)	ITU	Contact plates	TSA	350 / plate	*Staphylococcus*

a) Tryptic soy agar, b) Intensive care unit, c) Coagulase-negative staphylococci, d) Brain-heart infusion

It can be seen from Table 1.1 that the techniques used are wide ranging, making comparison between studies difficult. Variable conditions and differences in the type of information gained from sampling mean that, as yet, there is no single method used for all applications (46). There is a need for standardisation of sampling techniques that can be used to assess the microbial community in an area and possibly predict risk to human health. Once risk has been assessed, then action can be taken to prevent disease. Quantitative assessment of air and surface microorganisms is important in indoor areas where people gather, as high concentrations of pathogens may accumulate and outbreaks may occur, such as schools, hospitals and prisons.

1.3 Indoor microbial source and distribution

Despite not knowing the overall composition of microbial communities in indoor spaces, the origins of bacteria which may be present is largely understood. Although it may appear obvious, it has only been recently shown that outdoor air and human occupancy are among the major contributing factors to the presence of microorganisms indoors (47).

1.3.1 Human microbial sources

The human body is host to many varied microbial populations which are mostly non-pathogenic and in some cases as in the gut, for example, can be beneficial. However, the body can also carry microorganisms which have the potential to be pathogenic if

the right conditions arise. A number of these potential pathogens can be transmitted to other people and into the environment through coughing, sneezing, talking and movement. The main source of microbial particles in an indoor environment is known to be the presence of humans (10), (35), (48–52). It is estimated that the human body sheds approximately 10^7 skin particles per day (51), which are released into the environment and may carry microorganisms. Particles from the respiratory tract can attach to the skin or clothing via coughing, sneezing or talking and become aerosolised or involved in contact transmission from surfaces (50). Microorganisms normally associated with the skin or gastrointestinal tract may also spread to the environment through contact by hands (53) or vomiting (54), for example.

A sampling study conducted in Eastern Europe showed that indoor levels of bacteria were higher than in outdoor samples when large numbers of people were present (55). Ambroise et al. concluded the same; that exposure to viable airborne bacteria is much higher in the indoor environment and increases with human occupancy. In contrast, fungal spores do not often originate from humans and were found in the same study to be at much higher concentration outdoors than indoors (52).

Along with microorganisms which are part of the normal human microbiota, the body can serve as a source of transient microbiota, carrying those species that are not normally resident (53). Hands are often the main source of these transient species and can carry them from one location to another.

The normal human microbiota contains a number of bacteria which have the potential for pathogenicity (Table 1.2). Bacterial pneumonia, for example, is most commonly caused by *Streptococcus pneumoniae* and currently accounts for the majority of respiratory infection-related deaths and illness in England (56). *S. pneumoniae* is carried in the nasopharynx of approximately 60 % of healthy pre-school children (57) and rates of colonisation have been shown to be important in the spread of the organism in day-care centres (58). Concentrations of viable bacteria can be high in indoor settings and this can be further increased depending on the type of building, ventilation rates and other environmental factors.

Table 1.2: Examples of bacteria and their approximate rates of prevalence as normal human commensal species.

Species	Skin	Naso-pharynx	Mouth	Lower GI tract	Anterior urethra	Vagina
Staphylococcus epidermidis	100 %	100 %	100 %	25 %	100 %	100 %
Staphylococcus aureus	25 %	20 %	25 %	100 %	<5 %	25 %
Streptococcus mitis		25 %	100 %	<5 %	25 %	25 %
Streptococcus salivarius		100 %	100 %			
Streptococcus mutans		25 %	100 %			
Enterococcus faecalis		<5 %	25 %	100 %	25 %	25 %
Streptococcus pneumoniae	<5 %	60 % children	25 %			<5 %
Streptococcus pyogenes	<5 %	5-15 %	25 %	<5 %		<5 %
Moraxella catarrhalis		28-100 % children 1-10 % adults				

Species	Skin	Naso-pharynx	Mouth	Lower GI tract	Anterior urethra	Vagina
***Neisseria* sp.**		25 % - 100%	25 %		25 %	25 %
Neisseria meningitidis		<3 % children 24-37 % teens <10 % adults	25 %			25 %
Escherichia coli	<5 %	5-25 %	25 %	100 %	25 %	25 %
***Proteus* sp.**	<5 %	25 %	25 %	25 %	25 %	25 %
Kingella kingae		18 % children				
Pseudomonas aeruginosa		<5 %	<5 %	25 %	<5 %	
Haemophilus influenzae	<5 %	75 %	25 %			
Haemophilus parainfluenzae		77-100 %				
***Bacteroides* sp.**				100 %	25 %	<5 %
***Lactobacillus* sp.**		25 %	100 %	100 %		100 %
***Clostridium* sp.**			<5 %	100 %		
Clostriduim tetani				<5 %		
Corynebacteria	100 %	25 %-100 %	25 %	25 %	25 %	25 %
Mycobacteria	25 %	<5 %	<5 %	25 %	25 %	
Actinomycetes		25 %	25 %			
Spirochetes		25 %	100 %	100 %		
Mycoplasmas		25 %	25 %	25 %	<5 %	25 %

Adapted from Todar (59). GI: Gastrointestinal

1.3.2 Other sources of microorganisms

Whilst the predominant source of microorganisms in an indoor environment is its human occupants, other factors can contribute to microbial content. For example,

microorganisms from the outdoor environment will be introduced via outdoor clothing, shoes and open windows. Outdoor microorganism composition is influenced by the generation of aerosols during agricultural practices, waste water treatment, composting, animal husbandry and from landfill sites, soil and water bodies (60), (61). As such, it could be expected that a proportion of the indoor microbial community is also derived from these sources. The absence of curtains and regular floor cleaning have been shown to increase levels of outdoor bacteria in hospitals (50) and household environments (62). In a recent project screening residential properties, Dunn et al. have shown that the presence of pet dogs significantly contributes to the indoor microbiota. They demonstrated that differences in bacterial diversity between homes was mainly attributed to the presence or absence of a dog (63). This may also apply to other types of pet but this has yet to be reported.

Building work, contaminated furnishings, ventilation systems and overall building design can also influence the type of microorganisms found in indoor environments and alter the microbial profile (4), (10). Bacterial diversity has been shown to be lower in mechanically-ventilated rooms than in those that use open windows (4). Sink drain biofilms have also been shown to contribute to air contamination on hospital wards. Gilbert et al. found *Stentrophomonas maltophilia, Enterobacter cloacae* and *Sphingomonas paucimobilis* present in both air samples and in sink drain biofilm swab samples taken from the same ward (64). Building design and engineering has begun to be recognised as an important factor driving the microbial

ecology within a space (4), (65). The correct use of engineering control systems such as ventilation and waste disposal is also paramount in reducing the risk of infection. When systems such as these are misused or poorly maintained, problems related to outbreaks and health can occur. For example, multi-drug resistant *P. aeruginosa* caused a hospital-wide, long-term outbreak between 2005 and 2011 at a London teaching hospital when drains became blocked due to inappropriate disposal of waste and the sluice room and macerator were misused (66).

1.3.3 Spatial distribution

The spatial distribution of microorganisms within an environment appears to be largely dependent on the type of materials present, the presence or absence of moisture and the touch frequency of surfaces, however, once again, there is inconsistency in reports found in scientific literature (67). The majority of research into spatial distribution of microorganisms has been conducted in hospitals or health-care facilities. This is likely due to the fact that there can be some kind of control and replication applied to the studies due to the relatively regulated nature of these spaces. Particular species have been associated with particular spatial distribution, for example *Burkholderia cepacia* is often found on nebulisers and dental equipment and *S. maltophilia* on wet surfaces and sinks (68). *Acinetobacter baumanii* has been isolated from hospital bed rails, keyboards, mattresses and curtains (11) and the type of material bed rails are made from has been shown to have an impact on the survival and transmission of methicillin-resistant *Staphylococcus aureus* (MRSA) (69).

Recently, a number of projects have been carried out in the US to determine the spatial distribution of bacteria found in indoor environments. In a series of publications, it has been demonstrated that different areas within public restrooms, residential kitchens and other areas of the home have distinct bacterial communities (63), (70), (71). These differences are attributed to the use of the particular area, source of the bacteria and the localised surface conditions. It is clear that indoor microbiomes are complex and can vary greatly within one room. Studies that provide a microbial map of indoor areas are of great importance as they can provide knowledge of sources of microorganisms and could possibly be used to track the spread of pathogens.

1.4 Factors influencing the indoor microbiome

1.4.1 Physical parameters

Physical parameters such as temperature, relative humidity (RH), ultraviolet light (UV), air pressure and ventilation type have been shown to have an effect on the survival of microorganisms in the environment (10), (72) and overall microbial community structure (4). As these parameters can fluctuate frequently, it could be expected that the microbial population may change accordingly. However, understanding of how these factors influence microorganism populations and their compositions is limited and can be conflicting. Some studies show no correlation between microorganism presence and physical parameters. Augustowska et al., for example, found no correlation between microorganism numbers and temperature, RH or atmospheric pressure in a year-long monitoring study of hospital air (38).

Others suggest a correlation does exist; Obbard & Fang found that numbers of airborne bacteria were positively correlated to RH but not to temperature on a hospital ward (35), whereas Tham & Zurami found that under experimental conditions, viable airborne bacterial concentrations were higher at 20 ^{O}C than at 26 ^{O}C (51). Petti et al. agreed that humidity correlates positively with MRSA survival time on surfaces (73). A recent study by Kembel et al. showed a significant relationship between RH and temperature and the community structure of bacteria in indoor air. They demonstrated a difference in the number of taxa present and relative abundance in rooms with warm dry air versus those with cool moist air. They also found no correlation between ventilation method and pathogen load when testing naturally and mechanically ventilated rooms; however, they found that increased airflow by either ventilation type did reduce load (4). This has also been demonstrated by other studies (74), (75).

1.4.2 Temporal factors

Temporal variation in microbial communities has previously been linked to meteorological events (76) and season (60), (77). In the indoor environment, where the effects of season or weather may be less pronounced, certain activities or occurrences may contribute to peaks in microbial recovery, for example increased occupancy (52), bed making (78), or vomiting episodes (79). Also, outbreak conditions within indoor environments are transient and could influence the microbial community composition. The presence of certain taxa within a room may be influenced by the microorganisms carried by previous visitors to that room. This

is something highlighted by studies that have found if previous occupants of a room were colonised or infected with microorganisms, subsequent patients are at greatly increased risk of becoming colonised or infected with that same microorganism (80), (81), (82), (83). It is important to have knowledge of the 'baseline' profile of an environment in order to be able to interpret data gathered over a temporal survey. The temporal nature of microbial communities in air especially, is acknowledged in the literature (84), (85), which highlights the need for more long-term studies in order to be able to understand the indoor environment more thoroughly.

1.4.3 Bacterial survival on surfaces

Fomites are defined as any object capable of carrying an infectious microorganism and include items such as clinical equipment, keyboards and toys, for example. Fomites and surfaces are known to contribute to the spread of infection in indoor environments (12), (73), (80), (86). The role of inanimate objects in microbial transmission was previously controversial and debated but the presence of microorganisms on environmental surfaces and objects is now widely recognised, though it is often difficult to understand the significance of this. Microorganisms involved in infection including Norovirus, *Clostridium difficile*, *Escherichia coli*, MRSA and *Pseudomonas aeruginosa* have been shown to survive on inanimate objects for long time periods (Table 1.3).

Table 1.3: Survival times on surfaces of some clinically-relevant microorganisms

Microorganism	Environmental contamination	Survival time
Influenza	Aerosolisation after sweeping, fomites, coughing and sneezing	24-48 hours (67)
Parainfluenza	Clothing and surfaces	10 hours on surfaces, 6 hours on clothing (87)
Norovirus	Persistent outbreaks, extensive contamination, aerosolisation	RNA survival 7 days, surrogate virus 40 days (11)
SARS-associated coronavirus	Positive cultures from the environment	24-96 hours on fomites (67)
***Candida* spp**	Fomites	3-14 days (88)
C. difficile	Extensive and air	Spores: 5 months ,vegetative: 15 min- 6 hours (81)
P. aeruginosa	Sink drains, biofilms on dry surfaces	33 days on plastic (89) 48 hours on dry surfaces (90)
MRSA	Extensive and air	4 months (73)
***Enterococcus* spp**	Extensive	48 days on surfaces (91)

1.5 Environmental sampling methods

1.5.1 Air Sampling

Air sampling can be passive, whereby particles are allowed to settle by gravity onto nutrient agar plates, or active, consisting of drawing air into a sampling device at a specified flow rate. Passive air sampling may provide an initial indication of levels of microbial contamination but is not quantitative or of use in assessing bacterial diversity and will therefore not be discussed further. Methods of active air sampling include sampling onto filters from which microorganisms are mechanically removed (92), impaction onto solid agar or liquid impingement (93).

Choice of sampler depends on study type, required sampling time, portability and level of noise generated by the sampler. When quantifying viable microorganisms from the air, the ability of a sampler to retain viability (bioefficiency) is important (94). However, relying on bioefficiency alone may result in underestimation of microorganisms present. A low proportion of total bacteria will actually be culturable (95), (96) and particularly high viability losses are reported when dealing with Gram negative bacteria (97), (98). When assessing the total diversity of microorganisms in the air, molecular downstream processing techniques may be more useful, therefore maintaining viability is not always a priority.

1.5.1.1 Impingers: collection into liquid.

Impingers are collection vessels which operate under a pump that draws air into a collection liquid. Some models claim to have a collection efficiency of almost 100 % and collection into liquid may allow for greater flexibility of sample analysis than onto agar (99). However, evaporation can occur at long sample durations and pumps can only be operated at a low flow rate of 12.5 l / min, the average human breathing rate, therefore restricting the number of microorganisms collected.

1.5.1.2 Impactors: collection on to a surface

Impactors can be single stage or multi-stage and collect particles onto nutrient agar plates. The 6-stage Andersen sampler (Figure 1.1) is designed to collect bioaerosols of varying sizes, separating them into 6 fractions as a model of the human respiratory

system (51). Collection efficiency has been previously validated in many studies (51), (97), (100) and despite the possibility of agar drying out at extended sampling times, the sampler is still commonly used.

The volume of air sampled is limited when using agar-based sampling equipment. Sampling larger volumes of air might be expected to produce more accurate results but should the total number of colony-forming units (CFU) collected on agar plates exceed 200, it becomes difficult to count individual colonies due to overlapping (49). This means that these samplers can only be used for short periods of time.

Figure 1.1: Andersen 6-stage cascade impactor sampler.

1.5.1.3 Filter samplers

A range of filter types have been previously used for the capture of airborne microorganisms. These include polycarbonate (PC), polytetrafluoroethylene (PTFE),

mixed cellulose ester and gelatine. A review by Peccia et al. found that PC filters were the most commonly used for air sampling (101). Comparison studies suggest that PC filters are more efficient than PTFE at collecting small viral phages during experimental aerosolisation (102). Filters have been shown to have a collection efficiency of close to 100 % for bacteria and viruses but despite this, sample loss may occur during removal of microorganisms from the filter prior to processing (103). Gelatine filters avoid these losses as they can be completely dissolved in a buffer meaning total collection of all material (104). They have also been shown to collect viable virus from the air (102).

The AirPort MD8® (Sartorius Stedim Biotech, Germany) is a portable air sampler that can be used with gelatine filters in order to collect airborne microorganisms (Figure 1.2). It allows the collection of large volumes of air at flow rates consistent with not being damaging to microorganisms. The sampler has been used successfully to assess the airborne microbial content in spacecraft assembly facilities (43).

Figure 1.2: Sartorius Stedim AirPort MD8® air sampler with gelatine filter.

1.5.2 Surface sampling

Environmental surface sampling can involve the use of swabs or agar contact plates to recover microorganisms (45), (105), (106).

1.5.2.1 Swabs

Overall microbial recovery using swabs tends to be low (< 25 %) as microorganisms become trapped within the swab and this means the technique is never truly quantitative (107). There are a variety of types of swabs and methods proposed for increasing recovery, for example, flocked nylon swabs and the type of wetting solution may increase microorganism release (105), (108). There is a lack of standardisation of swab type and method across the literature. It is difficult to

standardise pressure and pattern of swabbing and this is a recognised limitation of the technique. Swabbing is however widely used and can provide useful information regarding surface contamination and is suitable for molecular and culture-based downstream processing methods.

1.5.2.2. Swabbing and enrichment culture

Swabbing followed by enrichment culture is often used when carrying out environmental sampling for specific pathogens such as MRSA (109) and glycopeptide-resistant enterococci (GRE) (110) and also when isolating pathogens from patient samples (111–113). These enrichment steps reduce the time taken to obtain positive results and can allow the detection of low concentrations of pathogens. Brain-heart infusion medium (BHI) is suitable for the culture of fastidious microorganisms from a range of materials and is often used when conducting enrichment from swabs (43), (114).

1.5.2.3 Contact Plates

Contact plates are small petri dishes filled with nutrient agar, typically trypticase soy agar (TSA) and are generally considered to provide a better measure of viable surface microorganisms than swabs when low numbers are expected to be present (105). The agar is poured to sit with a slight protrusion from the rim so when pressed down onto a surface, it makes contact and bacteria adhere. Again, there are limitations to this method and it is only quantitative in as far as it can remove

microorganisms from a surface. Also, some bacteria may grow better on a particular type of agar than others, so the method is not useful for total diversity studies. Contact plates may however, avoid the losses that occur with some swabs as there is no intermediate step between surface and growth medium.

1.5.3 Post-sampling processing: culture methods

To determine total viable counts of microorganisms, culture-based methods can be used. Liquid from impinger samples can be plated out onto selective agar when assessing bacteria or *in vitro* cell cultures carried out if investigating viruses. Filters can be directly placed onto agar or microbes can be disassociated into fluid which can be plated out, or added to cell monolayers.

Identification of bacteria from cultures can be carried out in a number of ways. Pure cultures must initially be obtained, which involves picking colonies from a mixed population and sub-culturing until all colonies appear uniform. Morphological classification can be achieved by the use of the Gram stain (54), however, this technique only allows the visualisation of shape and staining characteristics. To identify bacteria further, methods relying on biochemical properties such as the API system (Biomerieux, France) can be employed. This system provides identification based on the ability of bacteria to produce enzymes, such as catalase, or to metabolise certain compounds. While useful for identifying clinical isolates, the API system may not be as relevant for environmental strains as reference databases

available are not as comprehensive for these types of bacteria. Identification of virus from culture is a far more complex procedure and different viruses require different cell types to propagate. Therefore, identification of viruses from environmental samples using cell culture would involve only attempting to isolate one or a small number of selected virus types. This technique is not suitable for the quantification or identification of all viruses present in an indoor environment.

1.5.3.1 Limitations of culture methods

It is recognised that some bacteria, particularly Gram negatives and viruses can be damaged by sampling methods, becoming unculturable (115), (116). This can lead to an underestimation of quantity and if a particular genus is more susceptible than others to damage, misrepresentation of diversity within a sample.

Culture-based methods of isolating bacteria from the environment often use a single non-selective medium such as TSA [27], which allows detection of a wide range of bacteria. Some species, particularly some Gram positives, grow rapidly and may out-compete others on this type of agar, while other species may require complex nutrients and fail to grow on non-specific media. Many types of agar are available for the detection of different bacteria, such as mannitol salt agar for Gram positive bacteria and blood agar for haemolytic streptococci. In order to attempt to reliably isolate all types of bacteria from an area, multiple agar types can be used, as highlighted by a year-long study of a composting facility (117) but this can be time-consuming and expensive. Culture-based methods do not account for the large

proportion of bacteria which are unculturable. By definition this group will contain unknown and under-defined taxa and may lead to problems when trying to assess richness.

Aside from some taxa not being able to be cultured under any circumstances, a proportion of a bacterial population will be in a viable but not culturable state (VBNC) (15), (118), (119). This is thought to be induced in response to adverse environmental conditions such as low temperature in the case of *Salmonella* spp. or *Vibrio* spp., for example (15). This might be the case for bacteria in indoor environments, particularly pathogens as they will not be in their 'natural habitat'. The presence of VBNC bacteria may again lead to an underestimation of numbers if relying on colony counts on agar media. These bacteria may still pose a risk in an indoor environment and therefore must be considered when choosing a sampling strategy to assess the indoor microbiota.

Technical difficulties such as the overlapping of colonies on culture plates can also lead to the underestimation or poor interpretation of bacterial counts (115) (Figure 1.3). Despite these difficulties, a large proportion of investigations carried out that are concerned with microbial quantification and identification still rely on culturing to some extent. Culture can be a rapid way to identify areas that might be problematic in terms of cleanliness and risk in particular indoor environments, or to determine the presence of known culturable species. However, culture cannot solely be relied on when investigating the indoor microbiome and diversity within a space.

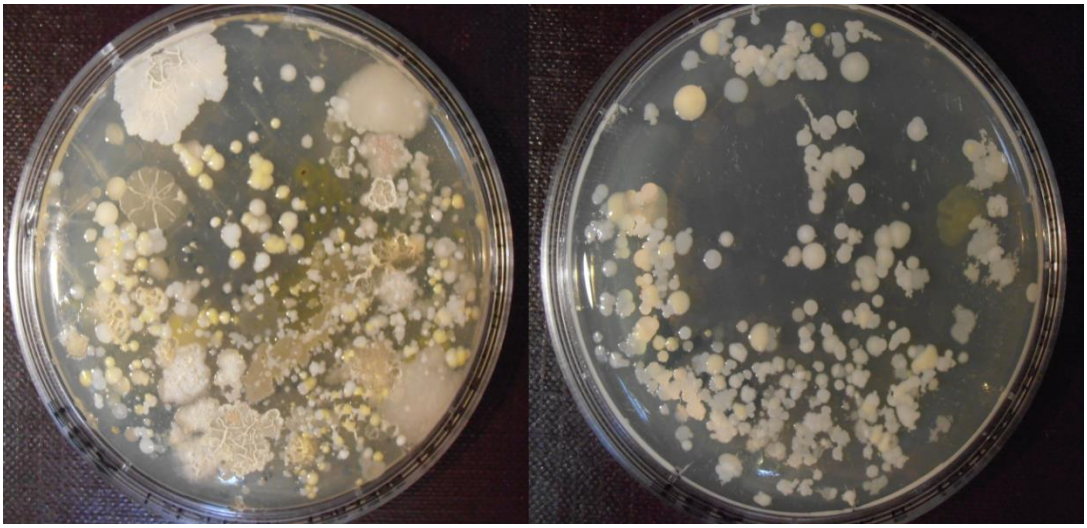


Figure 1.3: Tryptic soy agar contact plates showing overlapping and merged bacterial colonies leading to difficulty in counting.

1.5.4 Post-sampling processing: molecular methods

Knowledge and understanding of microbial communities has been greatly accelerated by the advent of nucleic acid analysis methods such as the polymerase chain reaction (PCR) and the development of deoxyribonucleic acid (DNA) sequencing in the 1970s (120). Currently, the most common approach for determining the phylogenetic and taxonomic structure of a bacterial community is to extract and purify DNA from samples, amplify a universal gene region; commonly within the 16S ribosomal ribonucleic acid (16S rRNA) gene, sequence the resulting amplicons and analyse the sequence data (121). This method has provided great insights into microbial communities from all environments and current rapid technological advances mean this knowledge is continuing to increase. Viral

identification is also possible without the need for culture and multiple virus types can be identified simultaneously from a single sample.

Nucleic acid amplification techniques provide advantages over culture techniques in that they are more sensitive, specific and rapid, allowing more data to be obtained quickly. They do not rely on the ability of the organisms to grow and can therefore detect viruses, unculturable bacterial species and those in a VBNC state.

The analysis of nucleic acids for microbial community studies involves extracting genetic material, amplifying a specific region of it to detectable or workable quantities and often, sequencing the fragments to identify the organism. The choice of method is fundamentally driven by the sample type but there are a number of issues to be considered that are universal to all sample types and investigations. Bias can be introduced at all stages when assessing microbial communities, from sampling through to data analysis and can have an impact on estimations of composition and abundance. Whilst some bias is currently unavoidable, steps can be taken to reduce it.

1.5.4.1 Nucleic acid extraction

A variety of protocols and kits are available for nucleic acid extraction and again, the method of choice is determined by sample type and to some extent, the post-extraction methods to be used. Very little, if any, comparative work investigating the

best methods for DNA extraction from indoor swabs and air samples has been carried out. Most comparative studies come from investigating human samples (121) or environmental samples such as soil and sediment (122).

DNA extraction involves disruption of the cell, known as cell lysis, followed by removal of cell membrane lipids and cellular proteins and purification of nucleic acids. Extraction techniques can introduce bias into diversity studies if cell lysis is incomplete, if compounds which inhibit downstream processing are co-extracted, or by loss or degradation of the nucleic acids. Silica column-based extraction methods or the addition of certain reagents such as bovine serum albumin (BSA), formamide or polyethylene glycol (PEG) are known to reduce the amount of inhibitors that are co-extracted (122), (123).

Disruption of microorganism cell walls and membranes can be achieved by physical, chemical or enzymatic lysis. It is recognised that Gram negative bacteria and viruses are more susceptible to lysis than Gram positive bacteria and spores. This can lead to overestimation of the number of Gram negative taxa in a sample, should the method not be sufficient to lyse all Gram positives. If the lysis step is too harsh, it could lead to the underestimation of Gram negative species due to damage to exposed nucleic acids. Viruses are also susceptible to damage by shearing and as such, methods may need to be adjusted if considering their recovery. The addition of a physical lysis step such as ‘bead-beating’ with glass or zirconia beads to extraction protocols is

often used to ensure efficient lysis of Gram positive bacteria and spores (101), (124), (125).

Physical lysis methods such as bead-beating often take place in the presence of a lysis buffer containing surfactants or detergents to simultaneously disrupt cell membrane lipids. Following cell lysis, a centrifugation step ensures separation and removal of cell debris. Nucleic acids are associated with proteins inside a cell and these must be removed to provide a pure solution. This is most often achieved by incubation with an enzyme such as protease or proteinase K. The nucleic acid is then purified to remove salts, detergents or other reagents used in the extraction process, as they may inhibit further analysis. This can be achieved by precipitating the DNA with ethanol, or by binding it to a column and washing a number of times.

1.5.4.2 End-point polymerase chain reaction

PCR is widely used to amplify a chosen region of DNA from target microorganisms and consists of three stages; 1) denaturation: double-stranded DNA (dsDNA) is split into two single strands, 2) annealing: small oligonucleotides that are complimentary to a chosen DNA region (primers) bind to the single-stranded template, 3) elongation: the chosen region of DNA is amplified. The reaction occurs in the presence of enzymes and components that are required for synthesis of new DNA strands. This results in the doubling of one dsDNA molecule to become two identical molecules. The process is repeated for a number of cycles and results in an exponential increase in the amount of DNA present. This large increase in

concentration of the target region allows it to be detected, usually by visualising on an agarose gel.

The ability of PCR to specifically detect microbial and viral DNA without the concern of culture makes it a powerful tool for the analysis of the indoor microbiome. The method can be targeted for the detection of specific microorganisms or for broad-range assays such as for the 16S rRNA gene, common to all known bacteria and Archaea and can be used alongside further processing to allow the detection of multiple bacterial taxa.

1.5.4.3 Nucleic acid-based analysis- quantitative real-time PCR

Quantitative real-time PCR (qPCR) allows the detection of PCR products as they are being generated, enabling quantification and avoiding the need for gel electrophoresis. Fluorescent dyes are included in the reaction mix, either in the form of SYBR® green, which binds to all dsDNA, or as probes, designed to be specific to the target region. As DNA concentration increases, so does fluorescence and the cycle number at which the fluorescence curve crosses a threshold value, the C_t, is used to quantify the amount of DNA in the starting sample compared to a standard curve.

qPCR is more sensitive than end-point PCR and can theoretically detect one gene copy in a sample. Probe-based assays are highly-specific and can identify small

target regions. Primers are used in the same way as for end-point PCR but another small oligonucleotide, which is complementary to a region inside the target DNA, is also included. The probe is synthesized with a fluorescent dye, known as a fluorophore, attached to one end and a compound which prevents it from fluorescing, a quencher, attached to the other end (Figure 1.4). The probe binds to the target region of nucleic acid and as the strand is copied by the PCR reaction, the fluorophore is cleaved from the probe. Fluorescence is detected by the qPCR instrument and is translated into DNA copy number.

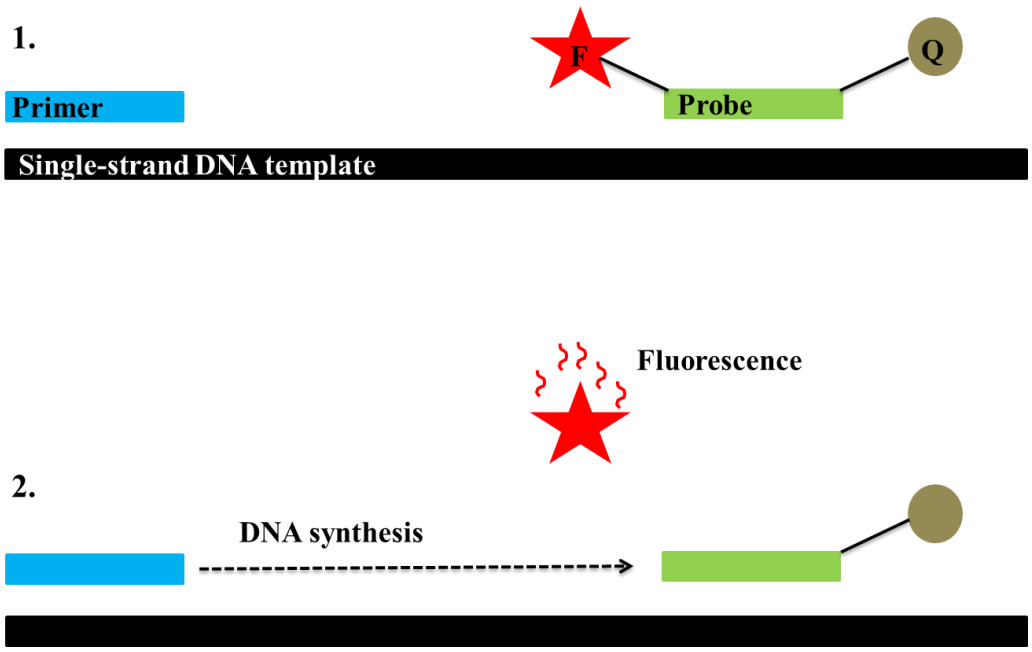


Figure 1.4: Diagram of primer and probe binding and fluorescence in qPCR.

qPCR can be used to identify and quantify specific microorganisms in samples from indoor environments and is a useful tool for this purpose (126), (127), (128). The routine use of specific molecular-based detection methods has the potential to have a

significant impact on public health. The rapid molecular detection of viruses such as the severe acute respiratory syndrome-associated coronavirus (SARS-CoV) or avian influenza from the air in prisons, schools and nursing homes, for example, could allow for physical barriers to infection to be implemented, preventing large outbreaks. This could also be true for bacteria such as *Mycobacterium tuberculosis* (*Mtb*) which is difficult to grow with samples taking weeks to show positivity. The detection of *Mtb* in the air with a rapid, specific test could allow the prevention of spread of infection as demonstrated by Vadrot et al. in a hospital environment (129).

The use of broad-range 16S rRNA gene qPCR to quantify total bacteria in indoor environments has so far not been widely investigated. The only information a broad-range qPCR assay such as this can give is an estimation of gene copy number present in a sample and whether it increases or decreases in relation to a parameter (130), (131). As such, it is usually carried out only as part of a larger investigation but may be useful as a quick indication of microbial contamination or 'bioburden'.

1.5.4.4 Limitations of PCR

End-point PCR as described, has practical detection limits between 8.0×10^4 and 4.3×10^6 gene copies and varies greatly depending on the DNA extraction method used and other factors (132). In order to detect a positive result, more than 4.3×10^6 gene copies must be present in the in the initial sample as DNA extraction is unlikely to ever be 100 % efficient. qPCR can overcome some of this limitation in that lower copy numbers are required for successful amplification. Inhibition of a PCR, be it

end-point or qPCR, results when a substance present in the sample prevents nucleic acid amplification and a false negative result is obtained. Environmental samples pose particular problems due to the presence of phenolic compounds, dust, pollen and humic acids which are known PCR inhibitors (129), (133). The appropriate DNA extraction method can clean up starting material and reduce or remove inhibitors. The PowerSoil® DNA extraction kit (MoBio, USA), for example, has Inhibitor Removal Technology® which claims to remove 100 % of environmental inhibitors but may not be suitable for all sample types. The addition of BSA, dilution and filtration have also been shown to remove inhibitors from environmental samples (123), (129). Care must be taken with samples of low microbial load, as the addition of BSA or other neutralising solutions may itself have an inhibitory effect and dilution of the sample to reduce this may result in the removal of target microbial sequences.

1.5.5 16S ribosomal RNA

Ribosomes are found in all living cells and are responsible for protein synthesis. They have 2 subunits and the genes that encode these subunits have been targeted for bacterial community analysis. The first molecular investigations attempting to characterise microbial biodiversity in environmental samples began in the early 1980's and utilised a component of the large ribosomal subunit; 5S rRNA (134). Ribosomal RNA (rRNA) was extracted, separated by polyacrylamide gel electrophoresis and analysed to provide phylogenetic information. However, this method proved only to be of value when analysing simple communities as, being

relatively short at 120 nucleotides long, the 5S rRNA could only provide limited taxonomic information.

The 16S rRNA gene was sequenced from *E. coli* in 1978 and the system of nomenclature referring to the nucleotide positions along the gene is still currently in use (135). The gene is approximately 1500 base pairs (bp) long and is present in all known species of bacteria and Archaea (136). The number of 16S rRNA gene operons varies between species and even between individuals within a species, ranging from 1 – 14 copies (137). 16S rRNA is a structural component of the small subunit of the ribosome and due to its ubiquity and key cellular function, the gene contains regions of highly conserved sequences. These sequences are interspersed with 9 hypervariable regions: V1 – V9, each of which show significant sequence diversity between bacterial taxa (138). The presence of highly conserved sequences allows the generation of universal PCR primers; designed to be complimentary to part of the conserved regions. These primers are designed to capture and amplify a portion of the 16S rRNA gene that contains at least one hypervariable region (Figure 1.5), allowing identification based on sequence differences.

Figure 1.5: Schematic of the 16S rRNA gene showing approximate locations of hypervariable regions V1 – V9 and examples of primer pair locations. Matching colours indicate complementarity. Adapted from Amann et al. (15)**.**

1.5.5.1 16S rRNA gene microbial community analysis

Microbial community analysis using the 16S rRNA gene began in the mid-1980's and was shown to be a rapid method to gain a large amount of information (139). Methods have traditionally used PCR of 16S rDNA followed by the generation of a genetic 'fingerprint' of the target microbial community. After PCR, the fingerprint

can be generated by a number of methods including those that utilise the sequence variation present in the amplicons such as; Denaturing Gradient Gel Electrophoresis (DGGE), Temperature Gradient Gel Electrophoresis (TGGE) and Terminal Restriction Fragment Length Polymorphism analysis (T-RFLP) or those that use the differences in length of generated amplicons such as; Ribosomal Intergenic Spacer Analysis (RISA) or length-heterogeneity PCR (LH-PCR). All of these methods allow a picture to be produced, either from an agarose gel or a chromatogram of fluorescent peaks, with each band or peak theoretically corresponding to one bacterial taxon. This allows the diversity of samples to be compared and intensity of bands or peaks theoretically corresponds to relative abundance within a sample.

The above methods are useful for detecting changes in microbial communities over time or in response to changing factors, however they have a number of limitations. Primarily, they do not allow the direct identification of bacterial taxa from samples. Bands can be cut from gels and sequenced but the 'gold standard' for identification of bacteria within a mixed sample has traditionally been through the generation of clone libraries. This technique is still in use but has limitations due to the relatively small number of fragments that can be analysed. NGS techniques are gaining popularity as they allow the theoretical sequencing of all 16S rRNA genes within a sample without the need for a cloning step. A review of NGS technologies is given in Chapter 6.

The use of the 16S rRNA gene for bacterial identification through sequencing has been pivotal in clinical research and practice. In 1991, the use of the technique allowed the characterisation of a previously unknown, unculturable actinomycete; *Tropheryma whippelii*. This was identified as the causative agent of Whipple's disease, a systemic infection commonly involving the duodenum and small intestine, resulting in fever, pain and diarrhoea (140). The use of 16S rRNA gene PCR for the detection of low copy number or unculturable bacteria in clinical samples was proposed in 2003 by Harris and Hartley and is currently in routine clinical use at GOSH and other NHS Trusts (141).

Sequencing of the 16S rRNA gene has been widely used to identify community structures of bacteria from indoor environments (142), soil and aquatic samples, (143), (144), (145) as well as for clinical diagnosis (141) and analysis of bacterial community structure related to periodontitis (146). It has also been demonstrated that 16S rDNA PCR-based methods are valid and useful when analysing the microbiological community within air (95), (101).

There is currently no consensus on which hypervariable regions of the 16S rRNA gene should be targeted to provide the most accurate bacterial identification. Comparative work has been carried out using many primer sets to determine the optimum hypervariable region to amplify. Chakravorty et al. reported that region V6 (nucleotides 986 -1043), despite being the shortest of the hypervariable regions at 56 bp, was the most heterogeneous, thus providing the best discriminating power (138).

They found that using the V6 hypervariable region, they could distinguish all but the most closely related of the Enterobacteriaceae; *Escherichia* sp., *Shigella* sp. and *Salmonella* sp. Wang et al. also found that the amplifying the V6 region could reduce the bias observed when amplifying other regions. Although, they reported only 91 % coverage when amplifying the V6 region, meaning that 9 % of bacteria in a sample were not identified (147). The V4 region currently appears to be the most popular choice for environmental studies, likely made so by the pioneering work of Caporaso et al. using Illumina MiSeq NGS technology (145). The V4 region is also used for NGS studies in the Earth Microbiome Project (29).

1.5.5.2 Limitations of 16S rRNA gene PCR

Copy number of the 16S rRNA gene varies among and within bacterial species and this can influence the sensitivity of a PCR reaction and can lead to over- or under-estimation of members of a bacterial community (137). Sequencing of the 16S rRNA gene classifies bacteria well at the genus level; however, there can be limitations when species-level identification is required. Some closely-related bacterial species share a high homology in the 16S rRNA gene sequence. *Neisseria* species, for example, show up to 96 % homology between pathogenic and non-pathogenic strains, therefore they are difficult to distinguish using this method (148), (149), (150). Identification of bacteria is dependent on the scope of the available database of known sequences (15) and can be lacking with regard to environmental isolates (151).

If a PCR primer is chosen to amplify a broad range of microorganisms, it is likely that even with the utmost care, contamination will be a concern. Some studies have shown that contamination of PCR assays can be caused by the PCR reagents themselves, especially *Taq* polymerase, as it is of bacterial origin (152), (153). The use of ultra-clean PCR reagents, reduction in number of PCR cycles, cleaning of equipment with DNAase (Ambion, UK) and separation of nucleic acid extraction, PCR set-up and post-PCR manipulation steps can reduce the chance of contamination occurring (154).

Bias can be introduced at all stages during molecular analysis of microbial communities, as previously discussed regarding cell lysis. The PCR step is recognised as one of the major factors contributing to biased results. Preferential amplification of certain templates over others is known to occur, along with the formation of chimeric sequences (15), (155). Chimeras are formed due to the elongation step of the PCR being terminated prematurely or by the annealing of highly conserved regions of 16S rDNA from different species (15) and can lead to incorrect identification in community analysis studies.

16S rDNA end-point PCR alone cannot be used to quantify relative abundance of bacterial taxa in a sample due to the differing numbers of gene operons between species. This also makes qPCR only semi-quantitative as multiple fluorescent signals do not always correspond to multiple bacteria.

1.5.6 Alternatives to 16S rRNA gene PCR for culture-independent bacterial identification

Other gene targets can be used to identify bacteria from samples. These include genes encoding for bacterial proteins such as topoisomerases which contain conserved and variable regions similar to those encoding 16S rRNA. Bacterial DNA gyrase and topoisomerase IV each have 2 subunits and the gyrB and parE subunits have been used as alternatives to 16S rRNA gene analysis when attempting to identify bacteria (156). These techniques have also been shown to be good at discriminating very closely-related bacteria such as species of *Pseudomonas*, *Bacillus*, and Enterobacteriaceae (157) and may therefore be a useful tool in clinical diagnostics.

Non-nucleic acid-based methods to identify bacteria have also been suggested in recent studies. Fox et al. suggest the use of modified matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOF MS) which uses peptides to differentiate bacteria based on peptide sequences (151). Each protein has a unique signal and can provide species-level identification (158). However, in the Fox et al. study, air was sampled onto agar culture plates prior to colony isolation and analysis, meaning that only the culturable fraction was identified. This is common when performing MALDI-TOF MS analysis, meaning that it may be more suitable for use in analysing factors such as drug-resistance after a molecular survey has been carried out (159).

1.6 Hospitals, nosocomial infection and the role of the environmental microbiome.

The study of the indoor microbiota is of importance in places where people are present and may be at risk of infection. Hospitals are unique environments that have been shown to drive microbial evolutionary processes, for instance, the emergence of vancomycin-resistant enterococci (VRE), as they contain a different sub-set of the human population and antibiotic use is frequent (160), (161). Hospitals by their very nature are temporary homes to those with infections, immobilised people who are at risk of contracting infections due to proximity to others and those who are more susceptible to infection than the general populous. The indoor microbiome of a hospital is therefore likely to be largely influenced by the people present and may contain a disproportionate number of pathogens compared to other environments. Certainly, information available at present regarding bacteria within hospitals is largely focussed on pathogens and their spread throughout these environments. The hospital indoor microbiota may have different characteristics than that of other indoor environments due to the populations present, cleaning regimes, use of antibiotics and overall building design. It has recently been discovered by Vickery et al., when investigating microbial contamination of a hospital, that bacteria can form biofilms on dry surfaces (162). This changes survival characteristics for species within these biofilms, making them on average 100-250 times more resistant to chemical and physical removal and possibly even up to 1500 times more resistant (162). The presence of biofilms must clearly now be born in mind when considering hospital environmental sampling. Other research has found that outbreak strains

within hospitals tend to be more resilient and resistant than sporadic strains (73). This research is beginning to aid our understanding of the hospital microbiome and may potentially lead to novel design and intervention strategies.

Studies investigating the hospital microbiota could shed light on not only what is present in the environment but how effective cleaning strategies are, where different species accumulate and what role the environment plays in cross-transmission of disease. They could highlight problem areas and discover design and usage issues within particular facilities (66). Currently, much research into hospital cleanliness attempts to use total aerobic counts on surfaces to quantify risk of infection (163), (164). However, without knowing what species are present, this is impossible and NGS-based community studies could fill this gap in knowledge.

1.6.1 Infection control and the hospital environment

1.6.1.1 Healthcare-associated infections

Healthcare-associated infections (HCAI) are defined by the Department of Health (DH) as;

"*Any infection by any infectious agent acquired as a consequence of a person's treatment by the National Health Service (NHS) or which is acquired by a health care worker in the course of their NHS duties*" (165).

HCAIs are an increasing threat to human health and are of particular concern in at-risk groups such as the very young (166), (167), elderly (168), those with indwelling

devices such as catheters (33), (169), or the immunocompromised (170). HCAIs adversely affect the quality of the UK health service, as well as being a problem worldwide and are estimated to cost the NHS over £1 billion per year (171). The emergence of multi-drug resistant strains of bacteria such as *Mtb* and *S. aureus* and new strains of viruses such as influenza has also led to increasing concern for healthcare workers (172). It is therefore understandable that a large proportion of research into environmental contamination and transmission of infection has been carried out in hospitals, particularly on wards (38), in operating theatres (50) and in clean rooms (98). However, these studies have a tendency to look at specific pathogenic organisms such as *Mtb* (173), *C. difficile* (174), or *A. baumanii* (175), or at specific locations that are perceived as higher risk. Despite this research, the problem remains that the relationship between levels of environmental microorganisms and incidence of infection is still not clear (10).

1.6.1.2 Hand hygiene

Infection control policies are often focused on hand-hygiene practices to reduce the spread of HCAI (53). The hands of those who come into contact with patients, such as doctors, physiotherapists, parents and nurses have been shown to become contaminated and are therefore a potential vector for the spread of microorganisms within the hospital environment. Rate of acquisition of bacteria on the hands of hospital staff was shown by Pittet et al. to occur at a rate of 16 CFU per minute of patient care (53).

Despite the focus on hand hygiene to reduce transmission of infection, hand hygiene policies have been shown to be difficult to enforce and are not always adhered to (176). This may imply that when it comes to reducing frequency of HCAI, other targets may be more effective. A recent long-term study showed that despite a reduction in environmental contamination and in hand-carriage of MRSA through an enhanced cleaning regime, the incidence of patient acquisition of MRSA was not reduced (177). This could suggest that the air may play a role in cross-transmission of this and other pathogens and it certainly highlights that there is more to learn regarding the mechanisms of spread of nosocomial disease. Environmental sampling for the presence of clinically important viruses is still in its infancy, as sampling for viruses poses unique challenges, as discussed. This means that viral distribution and environmental transmission risks are even less understood than for bacteria.

More research into the communities of microorganisms present in hospitals is vital as these are locations where people who are particularly vulnerable to infection spend time. Many patients are more susceptible to acquiring infections or becoming colonised with potentially pathogenic bacteria than healthy individuals (178), (179) and some are staying long-term in an environment where antibiotic-resistant and nosocomial microorganisms may be endemic (167), (180). As such, levels of environmental contamination should be monitored closely and should perhaps be maintained at lower levels than in other locations.

1.7 Clinically important pathogens

Many bacteria have the capacity to become pathogenic under the right circumstances. The extent of pathogenicity relies predominantly on bacterial factors but also on host factors, such as susceptibility, reduced immune-competence or the presence of in-dwelling devices. Bacterial drug-resistance is widespread and makes it difficult to control infection. Presented below is a brief overview of selected microorganisms that are of clinical concern, particularly in healthcare environments.

1.7.1 Enterobacteriaceae

The Enterobacteriaceae is a large family of Gram negative, rod-shaped bacteria containing many genera that are part of the normal human microbiota, particularly of the gut. It also contains numerous taxa of clinical concern, mainly due to their ability to acquire and share drug-resistance genes. Clinically-relevant taxa include *Klebsiella* spp., *Proteus* spp., *Enterobacter* spp., *Serratia* spp., and *E.coli*.

Gram negative Enterobacteriaceae, specifically those carrying drug-resistance genes, such as the extended spectrum beta-lactamase (ESBL)-producers, are the cause of a large proportion of HCAIs (181). Bacteraemia caused by resistant Gram negative bacteria has increased in the UK over recent years and is associated with increased mortality (182). *Klebsiella pneumoniae*, *E. coli* and *E. cloacae* cause a large proportion of nosocomial infection and are responsible for a wide range of clinical

presentations including urinary-tract infection (UTI), gastrointestinal infection, wound infection, bacteraemia and meningitis (181). ESBL-producing *E. coli* is also frequently isolated in community-acquired infections, particularly UTIs (183).

1.7.2 *Clostridium difficile*

C. difficile is Gram positive, anaerobic and spore-forming. It can be found in the normal gut flora of a low percentage of healthy adults (3 %), in a higher percentage of hospitalised adults (20-30 %) (184) and asymptomatic carriage in the guts of neonates is common (185). Clinical presentation of *C. difficile* infection is most commonly in the form of acute diarrhoea but infection can lead to severe colitis resulting in death (185), (186). Transmission predominantly occurs via the faecal-oral route but episodes of diarrhoea can lead to heavy localised environmental contamination and evidence also suggests that spores can become airborne and deposit on surfaces further from the bed space (174). *C. difficile* colonisation usually occurs as a result of either cross-infection or the use of antibiotics which allow proliferation in the gut. Spores are highly resistant to environmental conditions and can persist for up to 5 months (87).

1.7.3 *Acinetobacter* species

Acinetobacter spp. are Gram negative bacilli, found in soil and on the human skin, oropharynx and perineum as part of the normal microbiota (186), (187). *A. baumanii* is the most frequently isolated species in cases of infection but other species such as

A. lwoffii, *A. johnsonii* and *A. soli* have also been associated with disease (187), (188).

Pneumonia and lung infections are the most common presentations of *Acinetobacter* infection but a wide range of other infections can occur such as endocarditis, UTI, wound sepsis and bacteraemia (189). *A. baumanii* is one of the most common causes of neonatal bacteraemia and meningitis (190). Multidrug-resistant strains are commonly reported and are associated with poor clinical outcome and increased mortality (191), (192).

Acinetobacter spp. are resistant to some disinfectants and irradiation, form biofilms and can utilise many substrates to provide energy, therefore are found to persist in the environment, particularly in healthcare settings (187), (192), (193). Transmission routes are not clear but the environment is known to play a key role in hospitals, in particular. Recently, *A. baumanii* was isolated from the air in rooms of infected patients, indicating an air-associated transmission mechanism which might account for the rapid spread of pneumonia in outbreak conditions (194).

1.7.4 *Pseudomonas aeruginosa*

P. aeruginosa is a Gram negative aerobic rod and is considered an opportunistic pathogen of clinical relevance (59). It is ubiquitous in soil and water but can infect almost any tissue in the body when host defences are weakened, particularly in the

case of burns, cancer, cystic fibrosis and acquired immunodeficiency syndrome (AIDS).

Infection with *P. aeruginosa* can cause bacteraemia, cellulitis, pneumonia, UTI, endocarditis and neonatal diarrhoea (178). Nosocomial transmission has been shown to be linked to poor drainage and poor maintenance of water systems (66), (170). *P. aeruginosa* readily forms biofilms and is of particular concern in a hospital environment as it is resistant to temperature fluctuations, salts, antiseptics, disinfectants and many antibiotics (59,66). Other *Pseudomonas* species have also been associated with infection, such as *P. putida*, causing neonatal bacteraemia (195), (196) or *P. stutzeri*, causing endocarditis (197) but these are less frequently reported.

1.7.5 *Staphylococcus aureus*

S. aureus is Gram positive, found in the nasal passages of approximately 20 % of the human population and causes a range of clinical symptoms from superficial skin problems to more serious infections such as bacteraemia and pneumonia (186), (198). *S. aureus* is also implicated in around 50 % of surgical site infections in the UK (171). MRSA is resistant to a wide range of antibiotics and up to 50 % of *S. aureus* isolates in an area can be of this type (198). Rates of MRSA infection in the UK have been in decline over recent years, likely as a result of changing antibiotic prescribing practices and increased screening and decolonisation practices (171) but still remains a significant cause for concern, both in healthcare and community

settings. MRSA is rapidly shed into the environment, where it can survive for extended periods of time and is easily transferred to the hands and clothing of healthcare workers (13).

1.7.6 Drug-resistant enterococci

Enterococcus is a genus of Gram positive cocci and contains species of clinical concern including *E. faecalis* and *E. faecium*, both of which are present as part of the normal human gut microbiota. *E. faecium* is more commonly-associated with outbreaks (199) and causes endocarditis, UTI, wound infection and bacteraemia with infection rates increasing due to increased drug-resistance (200). VRE are widely reported and have a high level of resistance to multiple antibiotic classes (198).

Enterococci express a surface protein which allows the formation of biofilms and can colonise the skin and abiotic surfaces, changing rapidly between the two niches and demonstrate extended environmental survival (198). VRE rapidly contaminate surfaces and can survive for up to 2 months, causing prolonged and recurrent outbreaks (110). VRE transmission is thought to predominantly occur person-to-person, likely through unwashed hands and via fomites (83).

1.7.7 Viruses

A review of clinically important viruses is presented in Chapter 5.

1.7.8 Fungi

With the exception of *Aspergillus* spp. and some filamentous fungi of the order Mucorales, fungi do not tend to cause outbreak-scale infections. Yeast and fungal infections such as those caused by *Candida* spp., *Pneumocystis* spp. and *Cryptococcus* spp. can be of concern in immunocompromised patients. However, it was decided that, as the risk is relatively low and studies have been carried out into environmental contamination with some fungal species (201), (202), (203), (204), investigation into fungal contamination was outside the scope of this project.

1.8 Infection and paediatric patients

Pathogens of concern within healthcare environments currently include MRSA, VRE, *C. difficile*, drug-resistant and multi-drug resistant Enterobacteriaceae (MDE) such as ESBL-producing *Klebsiella* spp or *E.coli*, Norovirus (NV), *Acinetobacter* spp. and other antibiotic resistant bacteria (13), (161), (184), (182), (205). Within a paediatric environment, enteric and respiratory viruses such as NV, Adenovirus (AV), Rotavirus (RV) and respiratory syncytial virus (RSV), among others, represent additional challenges. Children tend to be more vulnerable to viral infections than older people due to the immaturity of their immune systems or the absence of vaccinations given at an older age (167), (206), (207). Children also have additional risk factors in the acquisition of disease; in addition to immunological naivety, they are often in close physical contact with their carers, visitors and other patients whilst in hospital, which may facilitate the spread of infection (167). Children may also

contribute to environmental contamination in different ways than adult populations. They can acquire disease early and may shed microorganisms, particularly viruses, for lengthened periods of time whilst remaining asymptomatic (167), (208), (209), (210), (211). Children are more likely to be admitted to hospital with infections that are related to community epidemics, such as respiratory and gastrointestinal viruses, and may therefore be a source of transmission within the setting of a vulnerable patient population.

1.9 Aims of the project

The aim of this project is to contribute towards the understanding of the microbiota of indoor environments. It has been demonstrated that knowledge of bioburden and compositions of microbial communities in indoor environments and what this might mean to those who use those spaces is incomplete. At the time of writing, long-term environmental sampling studies investigating microbial diversity of different indoor environments have rarely been carried out. This is particularly true of hospitals but also of comparative studies of different indoor spaces and those that use a variety of techniques.

The following thesis will be focussed on filling the identified gaps in the literature and the results gained from achievement of these aims will allow improved sampling of different indoor environments and increased knowledge of bacterial and viral communities within healthcare and educational settings. This study will allow an understanding of the microbial biodiversity within indoor environments and may

identify potential reservoirs of infection, providing practical applications for the knowledge obtained. This project introduces the concept of looking at an indoor environment as a distinct ecosystem.

The project aims to identify:

1) If levels of microbial contamination and taxonomic composition are different in healthcare and educational settings.
2) If viruses can be isolated from the air and surfaces in a healthcare environment and what factors may influence this.
3) If microbial numbers and community composition change over the course of a year on a new hospital ward and if the causes and consequences of any observed changes can be identified.

The achievement of these aims is described through the following thesis, the structure of which is described in Figure 1.6, below.

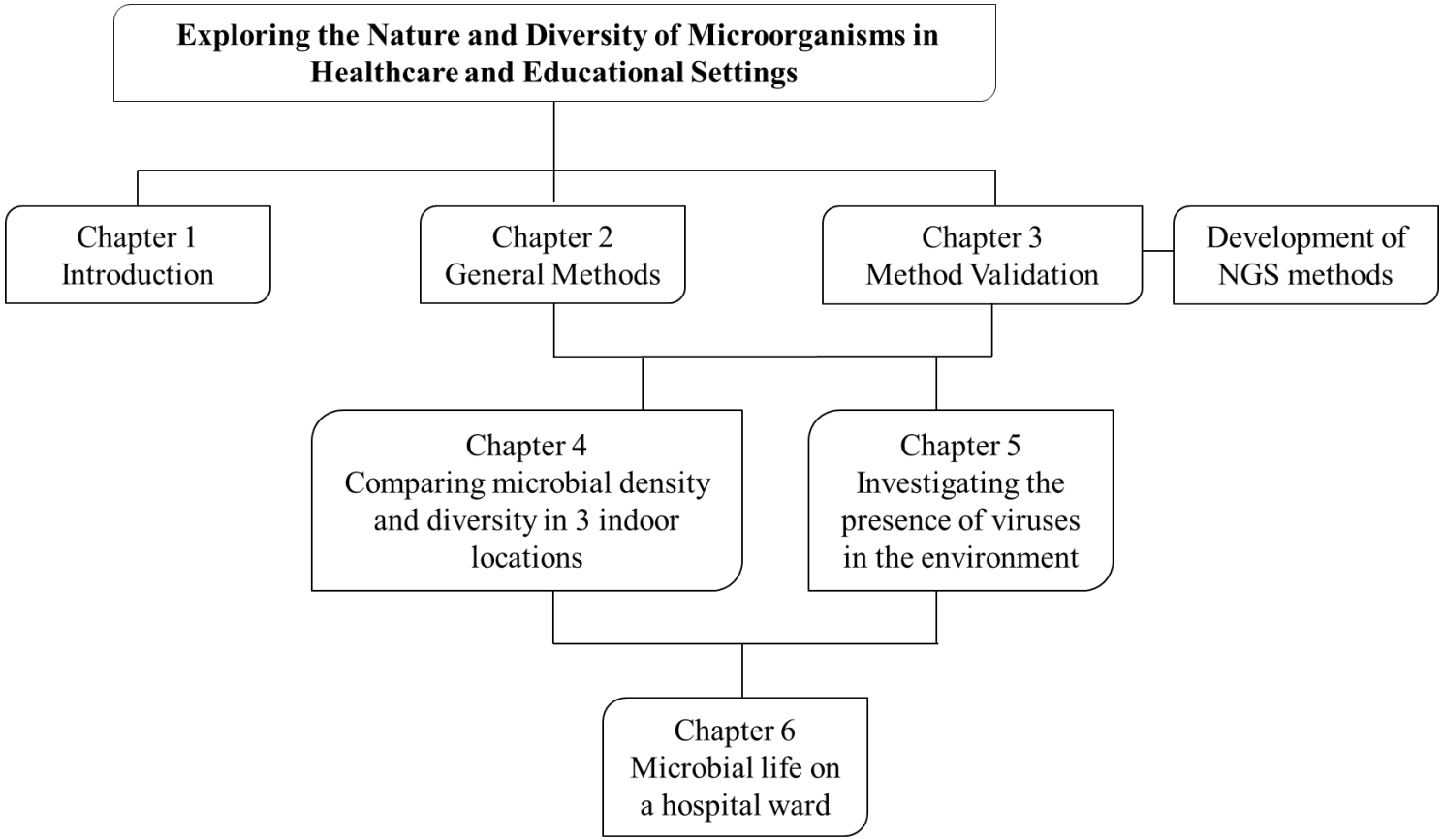


Figure 1.6: Flow diagram of thesis structure

2. Materials and Methods

The protocols detailed below use the best available and practicable techniques with a view to ensure that the highest possible quality of data was obtained. In order to determine the optimum environmental sampling and molecular methods to use for this study, validation experiments were carried out. These involved testing methods currently in use at Great Ormond Street Hospital (GOSH), the Eastman Dental Institute and from the literature to determine their suitability for the project. The results are presented in Chapter 3.

2.1 Safety, sterility and quality control

All laboratory techniques were carried out using good laboratory practice, aseptic technique where required and in accordance with local health and safety protocols, risk assessments and Control of Substances Hazardous to Health (COSHH) regulations. Where necessary, equipment, consumables and media were steam-sterilised in an autoclave at 121 ^{O}C for 15 minutes or in a dry oven at 180 ^{O}C for up to 2 hours. Plastic-ware, filters, pipettes and reagents for molecular analysis were exposed to UV-light at 254 nm for 30 minutes to eliminate the presence of residual nucleic acids.

2.2 Environmental sampling

Environmental sampling was based on the GOSH Operational Policy for microbiological screening of environment and staff (Appendix 1). Changes were made to locations and number of sample sites and screening of personnel was not conducted.

2.2.1 Contact plates

5 cm diameter TSA contact plates (Oxoid, UK) (25 cm^2) were used to gain a quantitative count of culturable bacteria present on surfaces. Plates were pressed onto surfaces with an even pressure for 10 seconds. They were then incubated at 37 ^{O}C for 48 hours prior to counting.

Plates have a grid on the reverse side to facilitate counting. Counting was conducted in a systematic fashion from left to right, top to bottom one square at a time and recorded as CFU per plate. Colonies that fell on the right and bottom of a grid line were not counted to ensure they were only counted once. The maximum colony count recorded was 300 CFU. Greater numbers than this formed a confluent layer.

2.2.2 Swabbing

2.2.2.1 Swabbing for molecular analysis

Cotton swabs were used and swabbing was carried out to the immediate right of where the contact plate had been taken. The swab tip was wetted in sterile, UV-irradiated AE buffer (10 mM Tris-Cl with 0.5 mM Ethylene-diaminetetraacetic acid (EDTA); pH 9.0) (Qiagen, UK) and run over the entire 10 cm^2 swab site horizontally, vertically and diagonally before snapping into a sterile, UV-irradiated microcentrifuge tube with 1 ml AE buffer. Swabs were frozen at -80 ^{O}C for subsequent processing.

2.2.2.2 Swabbing for enrichment

Sterile charcoal transport swabs (MWE, UK) were removed from the plastic sheath in which they were supplied and wet in sterile, UV-irradiated water (Invitrogen, UK). The swab was taken as above (2.2.2.1) and returned to the sheath. In the laboratory, swabs were snapped off into bottles containing 3 ml BHI broth (Oxoid, UK) and shaken briefly (114) . The tubes were incubated at 37 ^{O}C overnight. A loop-full (approximately 10 μl) was then streaked out onto blood or chromogenic agar plates (Oxoid, UK) which were further incubated for 24 hours at 37 ^{O}C. The bacteria were then identified using the methods outlined below.

2.2.3 Air sampling

The Sartorius MD8® Airport portable air sampler was used (Sartorius Stedim, UK) (Figure 1.2). Prior to sampling, gelatine filters were placed inside sterile petri dishes and exposed to UV-light for 30 minutes on each side. They were loaded onto a support mesh on the sampler, using sterile forceps at the sampling location. The air sampler was used at a flow rate of 50 l / minute to collect a total of 1000 l of air (1 m^3) (49). Filters were removed from the sampler in an aseptic manner into a sterile petri dish which was sealed with parafilm and transported back to the laboratory.

To ensure maximum recovery of airborne microorganisms from the filter and dissolution of the gelatine, filters were dissolved in 20 ml of pre-warmed, sterile, UV-irradiated phosphate buffered saline (PBS) (Invitrogen, UK). The dissolved filter was centrifuged at 40, 200 x g (15,000 rpm) for 20 minutes and the supernatant discarded (64). Any collected cells were then stored at -80 ^{O}C for subsequent processing. This procedure was carried out rapidly to prevent growth of any gelatine metabolising bacteria present in the sample.

2.2.4 Recording of environmental parameters

Temperature and relative humidity readings were taken at minute-apart intervals for the duration of each sampling procedure with HOBO® Data loggers (Tempcon Instrumentation Ltd., UK).

2.3 Culture methods

2.3.1 Culture for preparation of known numbers of bacteria

Cultures were prepared to provide known numbers of bacteria in suspension for DNA extraction to provide standards for molecular methods. Bacterial cultures were obtained from the American Type Culture Collection (ATCC). Strain numbers are given for each species in Table 2.1. Bacteria were sub-cultured onto blood agar (Oxoid, UK) and incubated overnight at 37 ^{O}C. The cultures were then processed using the Miles and Misra method described below (2.3.2).

Table 2.1: Bacteria obtained from the American Type Culture Collection

Bacteria	ATCC Number
Acinetobacter baumanii	BAA747
Elizabethkingia meningoseptica	13253
Enterobacter cloacae	700323
Klebsiella oxytoca	700334
Klebsiella pneumoniae	700603
Orchobactrum anthropi	BAA749
Proteus vulgaris	6380
Pseudomonas aeruginosa	BAA1744
Pseudomonas aeruginosa	9721
Shigella sonnei	25931
Staphylococcus aureus	9144
Stenotrophomonas maltophilia	17666

2.3.2 Miles and Misra method

Quantification of CFU present in bacterial suspensions was determined by the Miles and Misra Method (212). Overnight cultures of bacteria were prepared and 10-fold serial dilutions made by adding 100 µl of culture to 900 µl of PBS. 5 x 20 µl drops of bacterial suspension were pipetted onto agar plates from a height of 2.5 cm. Triplicate plates were prepared for each dilution. The plates were left to dry then incubated, inverted, for 24 hours at 37 ^{O}C. Plates of dilutions that showed discrete colonies of between 20-200 were counted (Figure 2.1). CFU per ml of original suspension was calculated by multiplying the average number of colonies across 3 plates of the same dilution by 100 (to achieve CFU / ml of diluted suspension) then multiplying by the dilution factor:

CFU per ml = Average number of colonies x 100 x dilution factor.

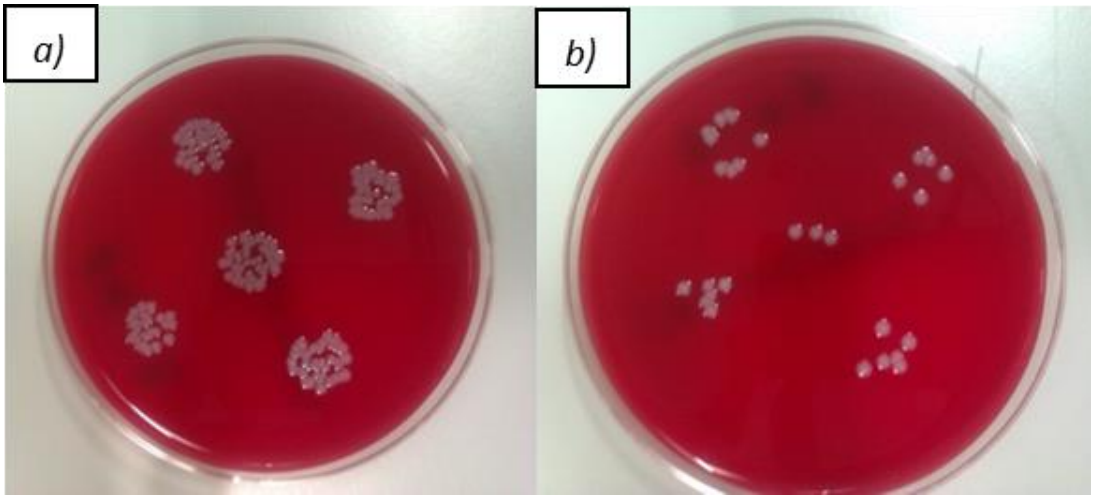


Figure 2.1: Miles & Misra preparation of bacterial suspension. a) Low dilution with merged colonies which are uncountable. b) Countable dilution.

2.4 Post-culture analysis

2.4.1 Gram staining

The following method was employed for the discrimination of Gram positive from Gram negative bacteria. A small smear of bacterial culture was applied to a glass microscope slide and passed through the flame of a Bunsen burner to fix it to the slide. The slide was flooded with crystal violet (Sigma Aldrich, UK) and left for 1 minute. The slide was then rinsed briefly with a gentle flow of water, flooded with iodine and left for 1 minute. The slide was rinsed again very briefly in water and flooded with acetone / ethyl alcohol (50:50 v/v) for no longer than 10 seconds. Safranin counterstain was applied to the slide for 30 seconds and the slide was rinsed again and left to air dry. Once dry, the slide was observed on a light microscope,

under oil immersion (x 100 lens). Purple cells were recorded as Gram positive and pink as Gram negative (54).

2.4.2 Biochemical identification

Streptococci and staphylococci are both Gram positive cocci and can be difficult to differentiate by eye on a culture plate or by Gram stain. They can be differentiated by biochemical means. These tests were performed to confirm sequencing data, predominantly for the comparative study presented in Chapter 4.

2.4.2.1 Catalase test

A small amount of a bacterial colony was picked with a sterile toothpick. The colony was then dipped into a solution of hydrogen peroxide (H_2O_2). If bubbling occurred, this indicated the presence of the enzyme catalase which breaks down H_2O_2 to hydrogen and water and the colony was considered to be *Staphylococcus* (in conjunction with Gram stain results). An absence of bubbling was scored as catalase negative and if Gram stain showed chains of Gram positive cocci, was assumed to be *Streptococcus*.

2.4.2.2 Latex agglutination

In order to determine if the identified *Staphylococcus* was the *S. aureus* species, a Pastorex® Staph Plus latex agglutination test was performed (Alere Ltd, UK). A small portion of a colony was picked with a plastic stick provided and smeared onto

a test card. A drop of test solution was added and the smear and solution blended with the stick. At the same time the colony was blended with a negative control solution on a different part of the test card. The card was rocked gently by hand for 30 seconds and the results read by eye. Agglutination was taken to mean the bacterial culture was *S. aureus* (Figure 2.2).

Figure 2.2: Pastorex latex agglutination of *Staphylococcus aureus.* The left sample is negative and the right positive.

2.4.2.3 Freezing of bacteria

Bacterial isolates, either pure or mixed, were frozen down by picking colonies from the agar plate on which they were grown, into a mixture of sterile nutrient broth and 50 % glycerol. These stocks were frozen at -80 ^{O}C.

2.5 Nucleic acid extraction methods

2.5.1 DNA extraction from pure bacterial cultures

For the preparation of DNA from pure colonies, a boiling and bead-beating method was used. A loop-full (10 μl) of bacteria was taken from the agar plate and placed in a screw-cap 2 ml microcentrifuge tube (Sarstedt, Germany) containing 200 μl of 0. 1 mm diameter zirconia beads (BioSpec Products, USA) and 1 ml sterile, UV-irradiated molecular grade water (AppliChem GmbH). The cultures were then heated to 95 ^{O}C and vortexed in a Disruptor Genie machine (Scientific Industries, Inc.) to disrupt cell walls and membranes. The tubes were then centrifuged at 16, 300 x g (13,300 rpm) for 2 minutes and the supernatant was removed to a clean tube. The resultant suspension was used as a DNA preparation for further analysis.

2.5.2 DNA extraction from cotton swabs

DNA extractions from swabs were carried out using a modified Qiagen DNA mini kit column-based protocol. Briefly, swabs were thawed and vortexed for 30 seconds. 400 μl fluid from the swab sample was added to a 1.5 ml screw capped tube containing 40 μl proteinase K, 200 μl 0.1 mm diameter silica beads, 400 μl Qiagen buffer ATL and 20 μl mouse cells to serve as an extraction / internal positive control (IPC) and qPCR inhibition control. The tubes were then vortexed on a Disruptor Genie machine for 10 minutes to disrupt cell walls and membranes and placed on a shaking heat block at 56 ^{O}C for 1 hour at 900 rpm.

The tubes were centrifuged for 1 minute at 6,000 x g (8,000 rpm) and the supernatant removed to 2 fresh tubes per sample (approximately 400 µl each). 400 µl Qiagen buffer AL was added to each tube and the tubes vortexed for 15 seconds. They were then incubated at 70 ^{O}C for 10 minutes with shaking at 900 rpm. 200 ml ethanol was then added to each tube, followed by vortexing again for 15 seconds. The lysate was then applied to the Qiagen column supplied with the kit. Each sample had 2 tubes, so one tube of lysate was applied to the column which was centrifuged at 6,000 x g (8,000 rpm) for one minute then the remaining lysate was applied to the same column and centrifuged again to bind the DNA.

The columns were then washed with 500 µl Qiagen buffer AW1 and centrifuged as above, followed by a wash with 700 µl Qiagen buffer AW2 and centrifuging again as before. The columns were finally washed with 700 µl ethanol and centrifuged again before a final centrifugation at 16, 300 x g (13,300 rpm) for 3 minutes to dry the column. Columns were further dried by incubating at 56 ^{O}C with lids open for 3 minutes to ensure no carry-over of ethanol. Nucleic acid was eluted in 80 µl buffer AE and stored at -80 ^{O}C until subsequent processing.

Extraction controls were included for each extraction set carried out. This consisted of using sterile AE buffer instead of sample to check for any contamination of the extraction process.

2.5.3 DNA extraction from gelatine filters

Gelatine filters were processed as described in Section 2.2.3. Frozen cell pellets were thawed and resuspended in 1 ml sterile AE buffer. 400 µl of the resuspended pellet fluid was processed as above (Section 2.5.2).

2.6 PCR methods

2.6.1 16S rDNA end-point PCR

A number of primer sets and conditions were used to amplify the 16S rRNA gene. All primers were from Eurofins MWG Operon, Germany, deoxyribonucleoside triphosphates (dNTPs), standard *Taq*, molecular grade water and PCR buffer all Bioline, UK. Moltaq, Molzyme water and buffer were from VH Bio, UK.

5 µl of sterile, UV-irradiated AE buffer was used as a negative, no-template control (NTC) in every reaction and positive controls consisted of 1µl of genomic DNA from *Escherichia coli.* In order to determine if negative PCR reactions were truly negative, PCR inhibition controls were included as necessary. A duplicate tube was prepared for PCR with 4 µl template and 1 µl positive control DNA (*E. coli*). If a negative result was obtained, the PCR was considered to be inhibited and was diluted 1:10 and re-run. Thermocycling was carried out in a G-Storm 482 thermocycler (Labtech, UK). Conditions differed for different primer sets and are outlined below (Tables 2.2 – 2.4).

Table 2.2: Conditions for 16S rDNA PCR with primer set 27F / 1492R

Reagent	Stock Concentration	Final Concentration	Volume (μl) / reaction
PCR H_2O	-	-	34.25
NH_4 buffer	10 X	1 X	5
dNTPs	10 mM	100 μM	1
$MgCl_2$	50 nM	250 μM	2.5
27F	10 pmol / μl	0.2 μM	1
1492R	10 pmol / μl	0.2 μM	1
***Taq* polymerase**	5U / μl	1.25 U	0.25

Primer Sequences	
27F	AGA GTT TGA TCM TGG CTC AG
1492R	ACC TTG TTA CGA CTT

Reference	Weisburg (213)

Mastermix added	45 μl
Template added	5 μl
Gene target	16S rDNA
Amplicon size	1465 bp

Cycling conditions	
Number	30

Step	Temperature (^{O}C)	Time
Initial (x1)	95	5 minutes
Denaturation	95	1 minute
Annealing	54	1 minute
Elongation	72	1 minute
Final elongation	72	5 minutes

Table 2.3: Conditions for 16S rDNA PCR with primer set 8a/8b and 3R

Reagent	Stock Concentration	Final Concentration	Volume (μl) / reaction
PCR H_2O	-	-	36.4
Molzyme buffer	10 X	1 X	5
dNTPs	25 mM	200 μM	0.4
8A (16SFa)	20 pmol / μl	0.4 μM	1
8B (16SFb)	20 pmol / μl	0.4 μM	1
3R (16SR)	20 pmol / μl	0.4 μM	1
MolTaq	5U / μl	1.25 U	0.25

Primer Sequences	
8A (16SFa)	GCT CAG ATT GAA CGC TGG
8B (16SFb)	CTC AGG AYG AAC GCT GG
3R (16SR)	TAC TGC TGC CTC CCG TA

Reference	Harris & Hartley (141)

Mastermix added	45 μl
Template added	5 μl
Gene target	16S rDNA V2
Amplicon size	320 bp

Cycling conditions	
Number	32

Step	Temperature (^{O}C)	Time
Initial (x1)	94	3 minutes
Denaturation	94	30 seconds
Annealing	63	60 seconds
Elongation	72	60 seconds
Final elongation	72	5 minutes

Table 2.4: Conditions for 16S rDNA PCR with primer set 785F / 1175R

Reagent	Stock Concentration	Final Concentration	Volume (µl) / reaction
PCR H_2O	-	-	37.4
Molzyme buffer	10 X	1 X	5
dNTPs	25 mM	200 µM	0.4
785F	20 pmol / µl	0.4 µM	1
1175R	20 pmol / µl	0.4 µM	1
MolTaq	5U / ml	1.25 U	0.25

Primer Sequences	
785F	GGA TTA GAT ACC CBR GTA GTC
1175R	AGC TCR TCC CCD CCT TCC TC

Reference	Bonder, (214)

Mastermix added	45 µl
Template added	5 µl
Gene target	16S rDNA V5-V7
Amplicon size	390 bp

Cycling conditions	
Number	32

Step	Temperature (^{O}C)	Time
Initial (x1)	94	3 minutes
Denaturation	94	30 seconds
Annealing	55	40 seconds
Elongation	72	90 seconds
Final elongation	72	10 minutes

2.6.1.1 Barcode 16S rDNA PCR for NGS

In order for PCR amplicons to be sequenced using the Illumina MiSeq NGS platform chosen for this study, primers had to be modified. Adaptor sequences to enable the amplicons to bind to a flow cell were attached along with individual barcode sequences to allow each sequenced amplicon to be identified (discussed in Chapter 6). Barcode sequences were obtained from http://www.earthmicrobiome.org/emp-standard-protocols/16s/ and adapter and linker sequences were designed to be specific for the chosen primer set in-house by Ronan Doyle.

The initial amount of template added was 2.5 µl in a 25 µl reaction. However, variation occurred within the samples and if amplification did not occur, the amount was adjusted sequentially to 3 µl, 5 µl, and 8 µl (reducing the water content accordingly) until amplification occurred. Reaction conditions are given in Table 2.5.

Table 2.5: PCR conditions for 16S rDNA barcode primers

Reagent	Stock Concentration	Final Concentration	Volume (µl) / reaction
PCR H_2O	-	-	17.13
Molzyme buffer	10 X	1 X	2.5
dNTPs	25 mM	200 µM	0.2
785F + adaptor	10 pmol / µl	0.4 µM	1
MolTaq	5U / µl	1.25 U	0.165

Primer Sequences	
785F + adaptor	AATGATACGGCGACCACCGAGATCTACAC TACCGGGACT TA GGATTAGATACCCBRGTAGTC
1175R	CAAGCAGAAGACGGCATACGAGAT BARCODE AACACGTTTT AA ACGTCRTCCCCDCCTTCCTC

Mastermix added	21.5 µl
Template added	2.5 µl
Gene target	16S rDNA V5-V7
Amplicon size	520 bp (390 + 130)

Cycling conditions	
Number	30

Step	Temperature (^{O}C)	Time
Initial (x1)	94	3 minutes
Denaturation	94	30 seconds
Annealing	56	40 seconds
Elongation	72	90 seconds
Final elongation	72	10 minutes

2.6.2 Quantitative real-time PCR

qPCR was carried out for specific or broad-range targets when it was necessary to either attempt to quantify microorganisms or isolate a particular species with a technique more sensitive than end-point PCR.

NTCs were included at least in duplicate by adding AE buffer in place of nucleic acid template. Accepted qPCR results had no amplification in NTCs and if a standard curve was included, reaction efficiency was within the range of 90 -110 % (215). If these criteria were not met, the assay was repeated.

Primers and probes were from Eurofins MWG Operon. All qPCRs were performed on an Applied Biosystems (ABI) 7500 Real-Time PCR cycler (Life Technologies, UK) apart from the 16S rRNA gene qPCR assay, which was performed on an ABI 7300 PCR System Cycler (Life Technologies, UK).

2.6.2.1 Standard curves

Standard curves were prepared, if appropriate, using the methods described in Sections 2.3.2 and 2.5.1 to quantify cell number and extracted DNA. A ten-fold dilution series of DNA was then prepared by diluting in AE buffer. Standard curves allow the quantification of DNA in test samples by comparing fluorescence produced by samples to the fluorescence of known copy numbers in the standard curve.

2.6.2.2 Mastermixes

A number of different commercially available master mixes were used, depending on the assay being carried out. An overview of each is given below.

Qiagen QuantiTect Master Mix

The QuantiTect Master Mix contains buffers to allow for efficient multiplexing of probe-based qPCR reactions, Uracil-N-glycosylase (UNG) to minimize carry-over contamination and HotStar *Taq* polymerase which requires activation at 95 ^{O}C prior to PCR. It also contains ROX passive reference dye which is required by ABI qPCR cyclers for fluorescence normalisation. Annealing and elongation steps are combined and fluorescence data are collected during this phase.

TaqMan® Universal PCR Master Mix

This master mix contains AmpliTaq Gold and provides an alternative to QuantiTect when multiplexing qPCR reactions. It has slightly different cycling conditions due to different components. It also contains ROX passive reference dye.

Power SYBR® Green Master Mix

Power SYBR® Green Master Mix (Life Technologies, UK) was used for 16S rDNA qPCR. It contains the reagents necessary for qPCR and AmpliTaq Gold, a Hot Start

Taq polymerase which is ultra-purified to minimise non-specific amplification. Power SYBR® Green Master Mix is reported by the manufacturer to be able to detect low copy numbers DNA from 1-10 copies per reaction.

2.6.2.3 Quantitative PCR reaction conditions

Reaction components and conditions for qPCR are given in Tables 2.6 – 2.9.

Table 2.6: qPCR conditions for *Staphylococcus aureus*

Reagent	Stock Concentration	Final Concentration	Volume (μl) / reaction
PCR H_2O	-	-	2.5
Sa-3-F Primer	10 pmol /μl	0.4 μM	0.5
Sa-3-R Primer	10 pmol /μl	0.4 μM	0.5
CoA Probe	10 pmol /μl	0.4 μM	0.5
QuantiTect Mastermix	2 X	1 X	14

Primer Sequences	
Sa-3-F	GTA GAT TGG GCA ATT ACA TTT TGA AGG
Sa-3-R	CGC ATC TGC TTT GTT ATC CCA TGT A
CoA Probe	FAM- TAG GCG CAT TAG CAG TTG CAT C - BHQ 1

Master mix added	18 μl
Template added	10 μl
Gene target	Coagulase gene
Amplicon size	78 bp

Cycling Conditions	
Number	45

Step	Temperature (^{O}C)	Time
UNG activation (x1)	50	2 minutes
Taq activation (x1)	95	10 minutes
Denaturation	95	15 seconds
Anneal / elongation	60	60 seconds

Table 2.7: qPCR conditions for *Klebsiella pneumoniae*

Reagent	Stock Concentration	Final Concentration	Volume (μl) / reaction
PCR H_2O	-	-	2.5
ent-dnak-F	10 pmol / μl	0.5 μM	0.5
ent-dnak-R	10 pmol / μl	0.5 μM	0.5
dnaK Probe	10 pmol / μl	0.5 μM	0.5
QuantiTect Mastermix	2 X	1 X	14

Primer Sequences	
ent-dnak-F	ACC TGG GTA CWA CCA ACT CTT GTG T
ent-dnak-R	GTC ACT GCC TGA CGT TTA GC
Probe	FAM- AGG ATG GTG AAA CTC TGG TWG GTC AGC C –BHQ 3

Master mix added	18 μl
Template added	10 μl
Gene target	Chaperone protein dnaK
Amplicon size	78 bp

Cycling Conditions	
Number	45

Step	Temperature (^{O}C)	Time
UNG activation (x1)	50	2 minutes
Taq activation (x1)	95	10 minutes
Denaturation	95	15 seconds
Anneal / elongation	60	60 seconds

Table 2.8: qPCR conditions for internal positive control

Reagent	Stock Concentration	Final Concentration	Volume (µl) / reaction
PCR H_2O	-	-	1
***Mus* F**	10 pmol / µl	0.5 µM	0.5
***Mus* R**	10 pmol / µl	0.5 µM	0.5
***Mus* Probe**	10 pmol / µl	0.5 µM	0.5
TaqMan Master Mix	2 X	1 X	14

Primer Sequences	
***Mus* F**	Unpublished – Not included at request of K. Harris
***Mus* R**	Unpublished
***Mus* Probe**	Unpublished

Master mix added	18 ml
Template added	10 ml
Gene target	Non-coding region
Amplicon size	73bp

Cycling Conditions	
Number	30

Step	Temperature (^{O}C)	Time
Taq activation (x1)	95	10 min
Denaturation	95	15 sec
Anneal / elongation	60	60 sec

Table 2.9: 16S rDNA qPCR conditions

Reagent	Stock Concentration	Final Concentration	Volume (µl) / reaction
PCR H_2O	-	-	9
SYBR Mastermix	10 X	1 X	12.5
785F	20 pmol / µl	0.4 mM	0.5
1175R	20 pmol / µl	0.4 mM	0.5

Primer Sequences	
785F	GGA TTA GAT ACC CBR GTA GTC
1175R	AGC TCR TCC CCD CCT TCC TC

Mastermix added	15 µl
Template added	10 µl
Gene target	16S rDNA

Cycling conditions	
Number	40

Step	Temperature (^{O}C)	Time
Initial (x1)	95	10 minutes
Denaturation	95	30 seconds
Annealing	55	40 seconds
Elongation	72	90 seconds
Dissociation (x1)	95	15 seconds
	60	30 seconds
	95	15 seconds

2.7 Gel electrophoresis

Different types of gel electrophoresis were carried out to visualise end-point PCR reaction products and are detailed below. E-gels® (Invitrogen, UK) offered a time advantage over standard gels and often provided better images in gel documentation equipment.

2.7.1 Standard agarose gels

5 µl of PCR product was mixed with 2 µl Crystal 5 X DNA loading buffer Blue (Bioline Reagents Ltd, UK) and loaded into wells of a 2 % (w/v) agarose gel containing 1 µl/100 ml Gel Red™ (Biotium, USA). Hyperladder I (Bioline Reagents Ltd, UK) was loaded into the first lane of each gel to assess the size of the PCR product. Gels were run in Tris/Borate/EDTA (TBE) buffer at 100 V for approximately 30 minutes. Gels were visualised under UV-light in a gel imager 300 nm transilluminator (Protein Simple, USA) and images captured using computer software (AlphaEase™, AlphaInnotech).

2.7.2 E-gels®

A pre-cast E-gel® cassette was clipped into a Power Base™ power pack (both Invitrogen, UK) and PCR product to a total volume of 20 µl was loaded into each well. If 5 µl PCR product was loaded, the remainder of the volume was made up by adding PCR-grade water. 5 µl of Hyperladder I was mixed with 15 µl PCR-grade water and loaded into the first well. The gels were run for 15–30 minutes. The E-

gels® used contained SYBR Safe® DNA stain within them and this allowed the DNA to be visualised in the same way as in Section 2.7.1.

2.7.3 Size Select™ 2 % E-gels®

Size Select™ E-gels® (Invitrogen, UK) allow PCR product to be collected after it has run through an agarose gel. This allows targeted recovery of DNA bands of required molecular weight for further downstream processing and eliminates the need for cutting sections from gels and purifying with a column. This method offers a time advantage over traditional gel-purification methods and less potential loss of DNA which may occur with a column.

Size Select™ E-gels® contain a proprietary fluorescent nucleic acid gel stain which allows the visualisation of anything above 1.5 ng of DNA per band to a maximum of 500 ng and is compatible with the Invitrogen iBase™ blue light transilluminator (excitation/emission at 490/522 nm).

20 – 25 µl PCR product was loaded into each well of a Size Select™ 2 % E-gel® and 10 µl (250 ng) E-gel® 50 bp ladder (Invitrogen, UK) was loaded into the central well. The gel was run on a pre-set program (Number 2) for 15.5 minutes. During this time, the PCR product was watched as it migrated through the gel. When the DNA band of the correct size reached a reference line, the run was paused and 25 µl of PCR-grade water was added to the empty collection wells.

The run was re-started and the bands of correct size were pipetted out of the well into a 1.5 ml low-bind Eppendorf tube (VWR, UK) as they migrated into the water. Size-selected PCR products were stored at 4 ^{O}C until required.

2.8 PCR purification

In order to clean DNA after PCR to remove salts and unincorporated primers or other PCR reaction components, PCR product purification was carried out.

2.8.1 Silica membrane column purification

PCR products were purified with either a Promega Wizard SV Kit (Promega, UK) or a QIAquick PCR Purification Kit (Qiagen, UK). DNA was applied to a membrane and contaminants were washed through and discarded. Bound DNA was washed twice and eluted in TE buffer (1 mM EDTA with 10 mM Tris) (Invitrogen, UK).

2.8.2 Sephadex™ column purification

GE illustra MicroSpin G-25 columns (Figure 2.3) hold Sephadex™ resin which contains pores that allow molecules of differing sizes to pass through at different times. Larger molecules are excluded first into an eluate which is the purified PCR product.

Columns were vortexed briefly to resuspend the Sephadex™. The cap was loosened by a quarter of a turn and the base of the column snapped off. The column was placed in a collection tube and centrifuged at 735 x g for 1 minute. The column was then placed into a fresh 1.5 ml tube and 50 ml PCR product was applied to the top. The column was centrifuged at 735 x g for 2 minutes and the eluate was stored at 4 ^{O}C until further use.

Figure 2.3: Sephadex™ column and collection tube for clean-up of PCR reactions

2.9 DNA concentration

The purity and concentration of DNA was checked prior to sequencing. This was carried out using 2 methods, initially for the comparative study presented in Chapter 4, the NanoDrop method was used. For the remainder of the work, the Qubit® method was used. This method is reportedly more sensitive than the NanoDrop and is recommended by Illumina for DNA quantification prior to NGS.

2.9.1 NanoDrop spectrophotometer

DNA concentration and purity were checked by measuring absorbance at 260 nm with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, UK). 1 μl AE buffer was added to the pedestal fibre optic cable to set a background reading. This was wiped away and 1.2 μl of DNA solution was applied to the machine. The concentration was recorded in ng / μl and purity assessed by recording the ratio of sample absorbance at 260 and 280 nm.

2.9.2 Qubit® 2.0 Fluorometer

The Qubit® Fluorometer (Invitrogen, UK) uses fluorescent dyes which bind only to the molecule of interest; in this case dsDNA. This makes it a more accurate measure of concentration, as single-stranded nucleic acids, proteins and other contaminating materials do not interfere with readings. DNA samples were prepared using the Qubit® dsDNA HS Assay (Invitrogen, UK), as described below.

2.9.2.1 Qubit® dsDNA HS Assay

For each DNA sample or standard, a total volume of 200 μl was prepared by diluting the sample or provided standard in Qubit® working solution. A range of DNA sample amounts were used from 1 μl to 20 μl depending on the amount of DNA solution available but final volume for the assay was always 200 μl. Working

solution was made by diluting the Qubit® dsDNA HS reagent 1:200 in Qubit® dsDNA HS buffer solution in a clean plastic tube.

For example, if 8 samples were to be measured, working solution for 8 samples and 2 standards was prepared: 10 tubes with 200 µl per tube yields 2 ml of working solution (10 µl of Qubit® reagent plus 1,990 µl of Qubit® buffer).

Standards were prepared for each new batch of working solution made. Standards were prepared by adding 10 µl Standard 1 to 190 µL of working solution and 10 µl Standard 2 to 190 µL of working solution and vortexing each for 2-3 seconds. DNA sample was added to the appropriate amount of working solution. For example, if using 1 µl this was added to 199 µl working solution. If using 10 µl, this was added to 190 µl working solution. Tubes were incubated at room temperature for 2 minutes the loaded onto the Qubit® Fluorometer and an absorbance reading was given. DNA concentration in the original suspension was calculated by the machine using the calculation below and recorded in ng / µl.

$$\textbf{Sample concentration} = \textbf{QF Value} \times \left(\frac{\mathbf{200}}{\mathbf{X}}\right)$$

QF value = the value given by the Qubit® 2.0 Fluorometer

x = the number of µl added to the assay tube

2.9.3 Agilent Bioanalyzer

In order to check final size and concentration of DNA libraries for NGS, the Agilent 2100 Bioanalyzer (Agilent Technologies, UK) was used. The DNA 1000 kit was used and prepared as follows, according to manufacturer's instructions. Gel dye mix was prepared by equilibrating DNA dye and DNA gel matrix to room temperature for 30 minutes, vortexing for 10 seconds and pulse centrifuging. 25 µl of dye was added to DNA gel matrix and vortexed for 10 seconds. The mixture was applied to a spin filter and centrifuged at 2240 x g for 15 minutes, after which the filter was discarded.

9 µl gel dye mix was then applied to a well of a DNA chip on the Chip Priming Station and pressure was applied by means of a syringe to distribute the gel around the chip. 5 µl of DNA marker was added to each sample well and 1 µl DNA ladder to the ladder well. The chip was vortexed for 60 seconds at 2400 rpm, loaded onto the Bioanalyzer and the analysis run started.

2.10 DNA Sequencing

2.10.1 Cycle (Sanger) Sequencing

PCR was performed, followed by gel electrophoresis of 5 µl PCR product (Section 2.7.1 or 2.7.2). If a band was visible on a gel, the remainder of the PCR product was purified and quantified using the methods given in Sections 2.8 and 2.9.

Concentrations of approximately 15 ng / μl were considered appropriate for Sanger sequencing of 16S rDNA PCR products.

For the comparative study (Chapter 4), 96 well plates containing 20 μl DNA per well at a concentration of approximately 15 ng / μl were prepared and sent to LGC Genomics (Germany) for sequencing. For the remainder of the study, samples were prepared and sequenced as follows.

2.10.1.1 BigDye® Terminator v3.1 Cycle Sequencing Kit

The BigDye® Terminator v3.1 Cycle Sequencing Kit (ABI, UK) was used to prepare PCR products for sequencing. Forward and reverse sequencing was always performed to give better quality data. Primers used depended on the primers used for PCR.

Sequencing primers were diluted to 2 pmol / μl in PCR-grade water. For 1 reaction, 1.5 ml PCR-grade water, 2.5 μl sequencing primer (2 pmol / μl), 1 ml BigDye® Sequencing Buffer and 2 μl Ready Reaction Premix were combined. A master mix of these components was made according to the number of sequencing reactions to be carried out. 7 μl of master mix was added to 0.2 ml PCR tubes (ABGene Thermoscientific, UK) and 3 μl of PCR product was added and mixed by pipetting. If a weak band was observed on the agarose gel performed after PCR, 4.5 μl of product was added and water omitted.

Tubes were loaded into a thermocycler and the following cycling conditions were applied: Rapid Thermal Ramp (RTR) to 96 ^{O}C, 96 ^{O}C for 1 minute followed by 25 cycles of RTR to 96 ^{O}C, 96 ^{O}C for 10 seconds, RTR to 50 ^{O}C, 50 ^{O}C for 5 seconds, RTR to 60 ^{O}C, 60 ^{O}C for 4 minutes, RTR to 4 ^{O}C and hold until ready to proceed.

2.10.1.2 Purification of extension products

Unincorporated dye terminators from the previous reaction were removed prior to sequencing. Samples were loaded onto a 96 well sequencing plate (ABI, UK), 5 µl 125 mM EDTA and 60 µl 96 % (v/v) ethanol (both Sigma, UK) was added to each well, the solution was mixed by pipetting and incubated at room temperature for 15 minutes.

The plate was centrifuged at 2,240 x g (4,680 rpm) for 30 minutes, then inverted on a paper towel and tapped, followed by a pulse centrifuge inverted on a paper towel to remove excess fluid.

60 µl 70 % (v/v) ethanol was added to each well and the plate centrifuged at 2240 x g for 5 minutes. The plate was inverted onto a paper towel and pulse centrifuged as before. Samples were then resuspended in 5 µl Hi-Di formamide and sequenced on an ABI 3130 capillary sequencer.

2.10.2 Illumina MiSeq Sequencing

2.10.2.1 Sample pooling

Prior to PCR for NGS library preparation, swab DNA extracts obtained for Chapter 6 were pooled into bed-space, non-bed space, and floor, sink and air samples. Bed space samples comprised objects near to the bed; chair arm, over-bed light, drawers (middle and bottom level), entertainment monitor, patient table, bed rail, extendable light, drug cabinet and trolley. Non-bed space items were found in the general ward area; window ledges (high, middle and bottom), bin, and alcohol or soap dispenser. Each time point therefore had a total of 8 samples; one pooled sample for each of bed spaces A – D, one non-bed space sample, one floor sample consisting of all floor samples taken, one sink sample and one air sample.

2.10.2.2 Standard MiSeq library preparation

Purified barcode PCR products were quantified by the Qubit® method (Section 2.9.2.1) and the concentration in nM of each sample was calculated using the following equation:

$$\textbf{Concentration (nM)} = \frac{\textbf{(}10^{6} \times \textbf{DNA concentration ng /µl)}}{\textbf{(649} \times \textbf{size of product)}}$$

This equation assumes the molecular weight of a single base pair of dsDNA is 649 g / mole. The size of the product was 520 bp (amplicon plus barcoded primers).

Each sample was then diluted to an equivalent nM concentration and 5 µl of each sample was pooled together to form the DNA library. The library was then quantified by the Qubit® method (Section 2.9.6.1) using 20 µl sample to 180 µl working solution. The pooled library was adjusted to a concentration of 2 nM and re-quantified using the Qubit®.

2.10.2.3 Denaturation of library

A fresh solution of 0.2 N sodium hydroxide (NaOH) was made by mixing 20 µl of 1 N stock (Sigma, UK) with 80 µl molecular grade water (VH Bio, UK). 10 µl of 0.2 N NaOH was added to 10 µl DNA library, vortexed and incubated at room temperature for 5 minutes. 980 µl HT1 buffer from the MiSeq Reagent Kit v2 (Illumina, UK) was added to dilute the DNA library to a concentration of 20 pM. The library was then further diluted to a loading concentration of 8 pM by adding 240 µl DNA library to 360 µl HT1 buffer.

2.10.2.4 Phi X control

Phi X control DNA (Illumina, UK) was diluted to 4 nM by adding 2 µl of a 10 nM stock solution to 3 µl H_2O. The Phi X library was denatured by adding 5 µl of 0.2 N NaOH to 5 µl Phi X, vortexing and incubating at room temperature for 5 minutes.

This was then diluted to a concentration of 20 pM by adding 980 µl HT1 buffer and diluted again to the same loading concentration as the DNA library. 100 µl Phi X was added to 900 µl DNA library to provide a final library with 10 % Phi X.

2.10.2.5 Preparation of MiSeq cartridge

The MiSeq reagent cartridge was thawed overnight at 4 ^{O}C. The foil of reservoir 17 was pierced with a long pipette tip. 600 µl of denatured DNA library with 10 % Phi X was loaded into the well and mixed gently by pipetting. 3.4 µl of 100 µM Index Sequencing Primer (5'-GAGGAAGGHGGGGAYGACGTTTAAAACGTGTT – 3') was added to reservoir 13, 3.4 µl of 100 µM Read 1 Sequencing Primer (5' - TACCGGGACT TA GGATTAGATACCCBRGTAGTC – 3') was added to reservoir 12 and 3.4 µl of 100 µM Read 2 Sequencing Primer (5-AACACGTTTTAAACGTCRTCCCCDCCTTCCTC – 3') to reservoir 14 and each was gently mixed by pipetting.

2.10.2.6 Loading the flow cell and buffer.

The flow cell (part of the MiSeq Reagent Kit v2) was removed from its buffer and washed with ddH_2O. It was dried with a lint-free wipe, ensuring that the inlet and outlet ports were clear and the surface was clean and smudge-free. The flow cell was loaded onto the MiSeq instrument. The chiller compartment was opened and wash bottle removed and replaced with PR2 buffer bottle. The reagent cartridge was loaded into the machine and the run started.

2.10.2.7 Low concentration DNA library preparation

Due to difficulties with sample preparation (detailed in Chapter 3), in addition to the protocol given in section 2.10.2.2, an alternative protocol for the preparation of DNA libraries was also used. The method, developed by Tony Brooks (Institute of Child Health) is designed for preparation of low concentration DNA libraries. The pooled library was quantified using the Qubit®. The appropriate volume of library was mixed with water and NaOH. This was then vortexed briefly, pulse-centrifuged and incubated at room temperature for 5 minutes. 2 μl hydrochloric acid (HCL) was then added and the tube was again vortexed and pulse-centrifuged. A library of 9 pM concentration was prepared in a final volume of 750 μl and Phi X spiked in as before.

2.10.3 Sequencing analysis and Bioinformatics

2.10.3.1 Sanger sequencing analysis

Sequence data were visualised using the Chromas LITE v2.01 software package (Technelesyium Pty Ltd, Australia) and compared to sequences in the GenBank database using the on-line BLAST program (http://www.ncbi.nlm.nih.gov). Sequences that showed 99–100 % similarity over the amplified region of the 16S rRNA gene were considered to be the same species and those showing 97–99 %, members of the same genus (216).

2.10.3.2 Bioinformatics

The Quantitative Insights Into Microbial Ecology package (QIIME) version 1.6.0 (www.qiime.org) was used (217). This contains all the relevant databases and programmes to analyse 16S rRNA gene sequencing data.

Paired reads were joined using FASTQ-Join. Sequences were de-multiplexed and quality filtered using the default pipline in QIIME and reads with a quality score (Q-score) of > 20 were selected. Operational Taxonomic Units (OTU) were picked with UCLUST which identifies 97 % difference as indicative of individual OTUs (218). OTU sequences were taxonomically classified by aligning to the Greengenes reference database, version 12-10, using PyNAST (145), (219). QIIME assigns taxonomy using the RDP Classifier 2.2 (220), using a naïve Bayesian classifier with a 0.80 confidence threshold. Minimum abundance thresholds were set to 0.005 % based on quality-filtering analysis reported by Bokulich et al. (2012) (221); all sequences representing < 0.005 % of the total reads for that sample were discarded.

2.11 Statistical analysis

Statistical analysis was carried out using the IBM SPSS Statistics software package version 21. Statistical tests used are referred to in individual results sections.

3. Method validation

3.1 Introduction

A wide variety of tools and techniques exist for sampling the microbial component of indoor environments. Samples can be taken from the air or surfaces and dust or other materials can also be collected. Sampling methods can create a bias in the numbers and types of microorganisms recovered and can interfere with downstream processing methods. Therefore, it is important to choose a strategy carefully and one that is relevant to the questions being asked of the study. An overview of different sampling methods was given in Chapter 1. The methods chosen for a study must provide an accurate as possible representation of microbial communities from the given environment. The selection of methods is dependent on the environment being assessed and the overall aims of the project. Post-sampling analysis methods are also wide-ranging and currently, largely involve the use of PCR. Various different PCR targets are used for microbial community analysis, commonly within the bacterial 16S rRNA gene. It is known however, that available primer sets can often fail to amplify some members of complex microbial communities (153), (222), (223). Therefore primer choice is a factor that must be considered when conducting community analysis work. The application of NGS to microbial community analysis studies is currently the most rapid way to identify large numbers of bacterial taxa in

a single sample. Due to the lack of published methods for the chosen platform at the time of conducting the study (discussed further in Chapter 6), method development for this project was necessary and is discussed below.

3.1.1 Chapter Aims

The aim of this chapter was to ensure the best data collection possible by determining optimum sampling and sample processing methods. The work aimed to test environmental sampling techniques for use in the project and post-sampling analysis methods. The specific aims are given below.

1. To compare recovery of viable bacteria and free DNA from a surface with different swab types.
2. To test the recovery of microorganisms from the air with the chosen air sampler.
3. To compare microbial cell lysis and DNA extraction techniques.
4. To ensure maximum DNA recovery from samples.
5. To choose the correct 16S rRNA gene hypervariable region for the project
6. To optimise PCR conditions for NGS
7. To develop a protocol for Illumina MiSeq NGS of samples obtained from indoor environments.

3.2 Development of sampling methods

3.2.1 Bacterial recovery and DNA yield from surfaces and filters

To determine which swab type would be used, sterile, UV-irradiated household tiles were inoculated with free bacterial DNA or whole viable bacteria and swabbed with either cotton swabs (MWE, Medical Wire) or flocked nylon swabs (Copan Flock Technologies, Italy). In order to test recovery from gelatine filters, filters were inoculated with known concentrations of microorganisms and nucleic acid extraction and amplification was performed.

3.2.1.1 Methods for testing bacterial and DNA recovery from swabs and filters

Swabbing tiles for recovery of viable bacteria

Both a Gram positive and a Gram negative bacterial strain were used due to the difference in cell wall characteristics and potentially different recovery rates and survival times on surfaces (67).

Overnight cultures of *K. pneumoniae* and *S. aureus* were prepared on blood agar plates and bacteria were removed from each plate into 5 ml of sterile PBS. A Miles and Misra dilution series was prepared (Section 2.3.2) to allow the quantity inoculated onto tiles to be determined. Tiles were divided into quarters (Figure 3.1)

to provide 4 replicates per condition. This suspension was applied in 5 x 50 μl drops on each quarter of the tile, using separate tiles for each organism. The drops were left to dry for 2 hours at room temperature. Once drops of bacterial suspension were dry, swab tips were moistened in sterile PBS and run over the whole swab site (tile quarter) horizontally, vertically and diagonally, before snapping off (cotton) or cutting off with sterile scissors (nylon) into a sterile microcentrifuge tube containing 1 ml PBS. The tube, with swab inside was then vortexed for 30 seconds.

100 μl of the suspension was serially ten-fold diluted in 900 μl PBS to a 10^{-8} dilution. A Miles and Misra dilution series was performed on blood agar to determine the CFU of viable bacteria recovered from the tiles. The remainder of the 900 μl swab fluid was stored at 4 ^{O}C for subsequent processing.

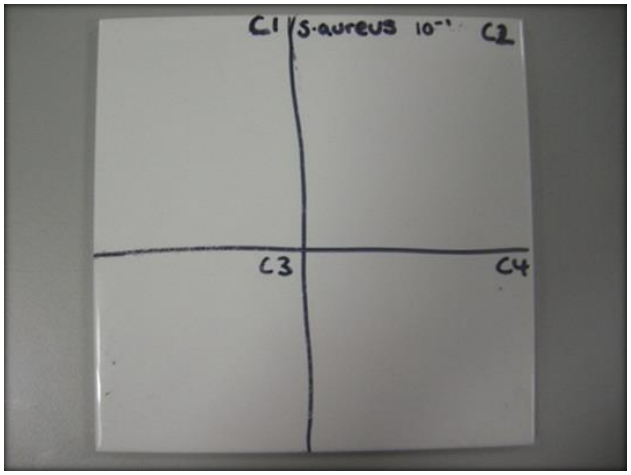


Figure 3.1: Example of a tile divided into 4 sections for inoculation of bacteria or free DNA.

Swabbing tiles for recovery of nucleic acid

DNA was extracted from bacteria as described (Section 2.5.2). Extracted DNA was diluted in AE buffer to 10^{-1}, 10^{-2} and 10^{-3} dilutions from the neat suspension and each dilution was inoculated onto tiles as above. Tiles were swabbed as above but using AE buffer as a wetting solution. After vortexing the swab in AE, the suspensions were used directly for qPCR as described in Tables 2.6 and 2.7. Blank swabs were also tested by qPCR to check for contamination.

Recovery of bacterial DNA from gelatine filters

The Sartorius MD8® air sampler was chosen for this project as it was deemed the most suitable given its portability, ease of use and quiet operation. Gelatine filters were chosen for use with this sampler due to the reported ability to recover 100 % of collected material. Over-night plate cultures of *S. aureus* and *K. pneumoniae* were

prepared on blood agar; bacteria were removed from each plate and diluted into 20 ml of sterile PBS. Miles and Misra plates were prepared as before to determine the total number of bacteria present.

Bacterial suspensions were adjusted to stock concentrations of 1 x 10^8. These were then serially ten-fold diluted in PBS to a concentration of 1 x 10^3 in a final volume of 1 ml. Gelatine filters were placed inside individual sterile 90 mm petri dishes in a class II microbiological safety cabinet (MSC) and UV-irradiated for 30 minutes on each side prior to use. Two filters were used per dilution per microorganism and blank, negative control filters were included (*n* =26). Each 1 ml suspension was applied to a filter and a further 9 ml sterile pre-warmed PBS was added to each dish. Dishes were sealed with Parafilm and placed inside an orbital incubator (Stuart, UK) with gentle agitation at 30 ^{O}C for 10 minutes to dissolve the gelatine.

Each suspension was transferred to a 15 ml tube and centrifuged at 4,500 x g for 20 minutes at 30 ^{O}C. Gelatine supernatant was poured off and the pellet resuspended in 1 ml sterile PBS. This was then centrifuged at 40,200 x g (15,000 rpm) for 5 minutes. The supernatant was removed and discarded and the pellet resuspended in 1 ml sterile, UV-irradiated AE buffer. DNA was extracted (Section 2.5.3) and qPCR for both organisms performed as described in Tables 2.6 and 2.7.

3.2.1.2 Results of bacteria and DNA recovery from swabs and filters

Recovery of viable bacteria

Recovery of viable bacteria was calculated by Miles and Misra dilutions of swab solution. Table 3.1 shows the recovery of each type of bacteria from tiles with each type of swab. Four replicates are shown for each swab type corresponding to four quarters of each tile. Different starting concentrations of each bacterial suspension were loaded onto tiles, therefore the recovery was adjusted to show the percentage of the initial inoculum recovered.

Overall recovery for both swab types and both bacteria was low. Mean recovery rates for cotton swabs were 4.6 % for *S. aureus* and 6.2 % for *K. pneumoniae* and for nylon swabs; 4 % and 9.3 % respectively. A two-sample independent Student's t-test showed there was no significant difference in recovery of viable bacteria between swab type ($P = 0.643$). Although there was a slightly better recovery overall of *K. pneumoniae*, this was again not statistically significant ($P = 0.6171$).

Table 3.1: Recovery of viable bacteria from tiles comparing cotton and nylon swabs.

Bacteria	Loaded (CFU / ml)	Swab type	Recovered (CFU / ml)	% Recovery
S. aureus	8.9×10^8	Cotton 1	5.6×10^7	6.3
		Cotton 2	2.3×10^7	2.6
		Cotton 3	4.8×10^7	5.4
		Cotton 4	3.8×10^7	4.3
		Nylon 1	3.2×10^7	3.6
		Nylon 2	4.8×10^7	5.4
		Nylon 3	2.4×10^7	2.7
		Nylon 4	3.7×10^7	4.2
K. pneumoniae	3.4×10^8	Cotton 1	1.2×10^7	3.5
		Cotton 2	3.0×10^7	8.9
		Cotton 3	1.0×10^7	2.9
		Cotton 4	3.2×10^7	9.4
		Nylon 1	3.0×10^7	8.9
		Nylon 2	3.4×10^7	10
		Nylon 3	3.4×10^7	10
		Nylon 4	2.9×10^7	8.5

Recovery of bacterial nucleic acid

Mean threshold cycle (C_t) values for each type of swab for each microorganism can be seen in Table 3.2. Figure 3.2 shows qPCR amplification plots of recovery of *S. aureus* (a, b) and *K. pneumoniae* (c, d) DNA at 3 decreasing concentrations when swabbing with cotton and nylon swabs. Standard curves were not included therefore this is not a quantitative measure of recovery; however a comparison can be made between swabs of the cycle number at which amplification for each dilution

occurred. This provides a relative estimate of the DNA present in each sample. A lower C_t value corresponds to more DNA being present.

Table 3.2: Mean threshold cycle (C_t) values for three dilutions of *S. aureus* and *K. pneumoniae* DNA recovered from tiles.

		C_t			
Bacteria	**Swab**	10^{-1}	10^{-2}	10^{-3}	**Negative**
S. aureus	**Cotton**	19.32	22.14	25.22	34.45
	Nylon	19.15	21.89	26.28	37.28
K. pneumoniae	**Cotton**	18.06	24.42	26.01	36.73
	Nylon	18.81	24.6	26.11	38.02

There was little difference between cotton and nylon swabs in recovery of DNA for either organism at each dilution. Cotton and nylon swabs both had a C_t value of 19 for the 10^{-1} dilution of *S. aureus* DNA and 18 for *K. pneumoniae* DNA at the same dilution, meaning that there was no difference in recovery of the two types of nucleic acid based on swab type. The 10^{-2} and 10^{-3} dilutions also had little or no difference in recovery between swabs.

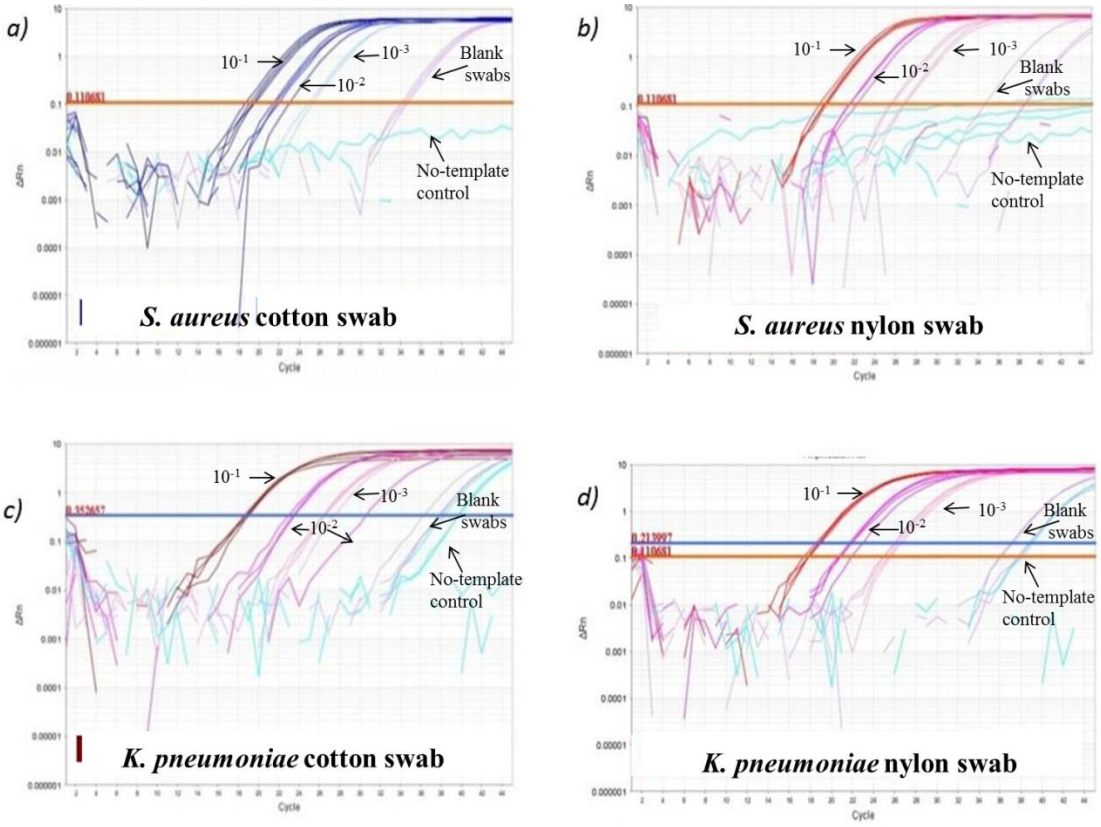


Figure 3.2: Amplification plots for bacterial DNA after swabbing.
a) *S. aureus* 10^{-1}, 10^{-2} & 10^{-3} cotton swab. b) *S. aureus* 10^{-1}, 10^{-2} & 10^{-3} nylon swab. c) *K.pneumoniae* 10^{-1}, 10^{-2} & 10^{-3} cotton swab.
d) *K. pneumoniae* 10^{-1}, 10^{-2} & 10^{-3} nylon swab.

DNA recovery from filters

Table 3.3 shows the mean C_t values and copy number for the DNA recovered from filters inoculated with *S. aureus* and *K. pneumoniae* DNA dilutions. There appeared to be less total loss at higher dilutions with *K. pneumoniae* than with *S.aureus*. At the 10^{-3} dilution, copy number was less than for the negative controls for both types of bacteria. Indicating that the limit of detection for each assay is approximately 10^4 gene copies, for this assay. The step-wise decrease in C_t was slightly smoother for

K. pneumoniae (Figure 3.3). Overall recovery appeared better for *K. pneumoniae* with higher C_t and copy number values being recovered than for *S. aureus*. However, both strains were able to be recovered from gelatine filters over a dilution series and detected using the qPCR methods used.

Table 3.3: Mean C_t values for DNA recovered from filters inoculated with *S.aureus* and *K.pneumoniae* DNA dilutions.

	C_t		Copy number	
Dilution	***S.aureus***	***K.pneumoniae***	***S.aureus***	***K.pneumoniae***
10^8	26	21	21984588	197356440
10^7	33	25	572759	20156341
10^6	33	28	416001	2706967
10^5	37	33	12925	131829
10^4	40	34	1399	4287
10^3	41	37	558	2680
Negative	41	34	1000	4125

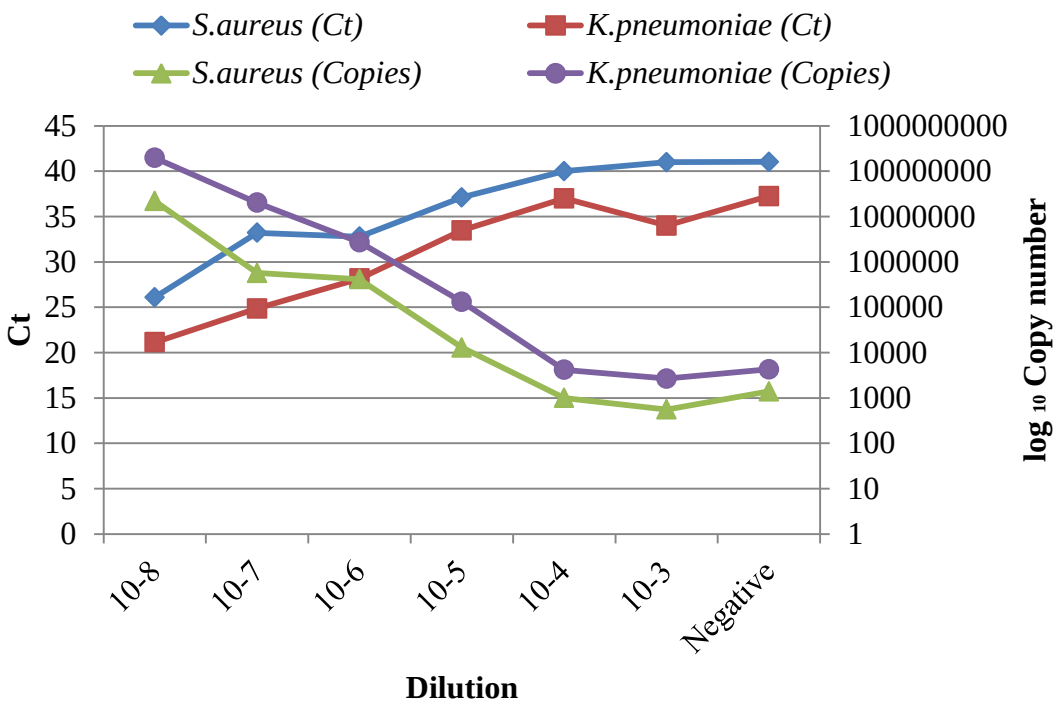


Figure 3.3: C_t and copy number for *S. aureus* and *K. pneumoniae* DNA extraction from filters over a dilution series.

3.2.1.3 Bacteria and DNA recovery discussion

Swab type

There has been suggestion in the literature that flocked nylon swabs allow for greater recovery of viable microorganisms from surfaces than do cotton swabs (105). This is due to the suggestion that microorganisms can become trapped in the fibres of cotton swabs and therefore less will be released into the swabbing solution for further processing, leading to an underestimate of microorganism numbers. This is reported not to happen with flocked swabs as they do not have tightly wound fibres which may trap microorganisms.

Based on the above experiments however, it was determined that no significant advantage would be gained by using flocked nylon swabs for the current investigations. Cotton and nylon swabs performed equally when recovering viable bacteria and free bacterial DNA from tiles. There was slightly better overall recovery of viable *K. pneumoniae* from tiles than *S. aureus*. Both *S. aureus* and *K. pneumoniae* have been reported to be able to survive on surfaces for months (67) and Gram negative bacteria have been shown to survive longer overall than Gram positives (90). The swab recovery rates compare with other published reports in that recovery is low (106), (107), (224).

Cotton swabs provide a 'general-purpose' swab and are easy to use in the field. Flocked nylon swabs have a plastic shaft which means they must be cut off into the microcentrifuge tube; this is difficult to do in the field and is a source of potential contamination. Cotton swabs have a wooden shaft which is easily snapped off and this reduces the risk of contamination. Currently, there are no commercially available flocked nylon swabs with wooden shafts. This fact combined with the data obtained during the validation experiments lead to the decision to use cotton swabs for sampling the environment for the remainder of the study. Overall recovery was low and this is accepted as a limitation of the method, however, the method is still widely used and is a standard method for infection control screening at Great Ormond Street and other NHS trusts.

Recovery of DNA from gelatine filters

There appears to be good recovery of bacterial DNA from gelatine filters at higher concentrations. At lower concentrations, particularly with *S. aureus*, sensitivity of the method begins to decrease. Given that bacteria are reported to be present in the air in high concentrations, the ease of use of the sampler and its relevance for use in the environments under investigation, it was determined that the loss of sensitivity at lower concentrations was acceptable.

3.2.2 Comparison of DNA extraction methods

Pure cultures of *S. aureus* and *K. pneumoniae* were tested and analysed by qPCR to determine the best DNA extraction method for the work.

3.2.2.1 Comparison of DNA extraction methods

Overnight cultures of *S. aureus* and *K. pneumoniae* were prepared as before (Section 2.3.1) and concentrations adjusted to 1 x 10^8 cells / ml. Cell suspensions were processed in duplicate using each of 4 methods; 1) AGOWA® mag magnetic bead kit (AGOWA® GmbH, Germany), 2) Modified Qiagen QIAamp DNA mini kit (Qiagen, UK), 3) crude heat lysis and 4) Phenol:Chloroform extraction. Nucleic acid extracts were then amplified by qPCR as before.

AGOWA® mag kit

The kit was used according to manufacturer's instructions. Briefly; cells were mechanically lysed by bead-beating with 0.1 mm diameter silica beads in the presence of lysis buffer and phenol. 10 µl magnetic beads were added to the sample along with binding buffer and the sample was incubated in a magnetic separator. Beads were then washed and separated twice before drying and eluting nucleic acid into AE buffer.

QIAamp DNA mini kit

The manufacturer's protocol for DNA extraction was followed with modifications. Briefly; 20 µl proteinase K, 10 µl mouse cell IPC, 200 µl 0.1 mm diameter silica beads and 200 µl Qiagen buffer ATL were added to 200 µl bacterial cell suspension. Samples were incubated for 10 minutes at 56 ^{O}C before bead-beating at full speed for 10 minutes on a Disruptor Genie (Scientific Industries, Inc.). 200 µl of 70 % ethanol was added and the lysate was mixed and applied to a Qiagen column. The protocol was then followed by washing the column twice prior to nucleic acid elution in AE buffer.

Crude DNA extraction by heat lysis

200 µl of silica beads were added to 500 µl of cell suspension. The sample was then heated to 95 ^{O}C for 10 minutes before bead-beating at full speed as above. The supernatant was removed and used as a crude nucleic acid extract.

Phenol: Chloroform extraction (P:C:I)

The cell suspension was centrifuged for 10 minutes at 16,300 x g (13,300 rpm). The supernatant was removed and the pellet was resuspended in 0.5 ml 120 mM phosphate buffer with 5% hexadecyltrimethylammonium bromide (CTAB). The solution was transferred to a sterile 2 ml screw-cap microcentrifuge tube containing 200 µl silica beads. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) (Sigma Aldrich, UK) was added and bead-beating carried out as above.

Tubes were cooled on ice and centrifuged at 16,300x g (13,300 rpm) for 5 minutes at 4 ^{O}C. The top aqueous layer was transferred to a new microcentrifuge tube. An equal volume of chloroform: isoamyl alcohol (24:1) was added and the solution mixed. Tubes were centrifuged again as above and the top layer was removed to a new tube. Two volumes of PEG solution was added and the solution was mixed and left for 1-2 hours at room temperature or overnight at 4^{O}C to allow the DNA to precipitate. Tubes were then centrifuged for 10 minutes at 16,300 x g (13,300 rpm), the supernatant was carefully removed and the pellet washed twice with 200 µl 70 % ethanol and centrifugation for 5 minutes. The pellet was left to dry for 20 minutes

and resuspended in 50 ml sterile, UV-irradiated TE buffer. The resultant solution was used as DNA template for further analysis.

3.2.2.2 Comparison of DNA extraction methods results

Gram positive bacteria

DNA from samples containing 9.5 x 10^8 *S. aureus* cells was extracted and processed in duplicate using each of the 4 methods described above. Amplification occurred around 5 cycles earlier with the column-based extraction method than for both the AGOWA® and P:C:I methods (Figure 3.4), meaning more DNA was present.

The crude extract was detected at around cycle 7 on the amplification plot but it was not a true amplification curve (Figure 3.4), indicating that there was either too much nucleic acid material in the sample or contaminating material was present. Replicate C_t values were almost identical for the column-based method but differed by 1 cycle for the P:C:I methods and by over 2 cycle for the AGOWA® kit (Table 3.4). All negative extractions had no amplification.

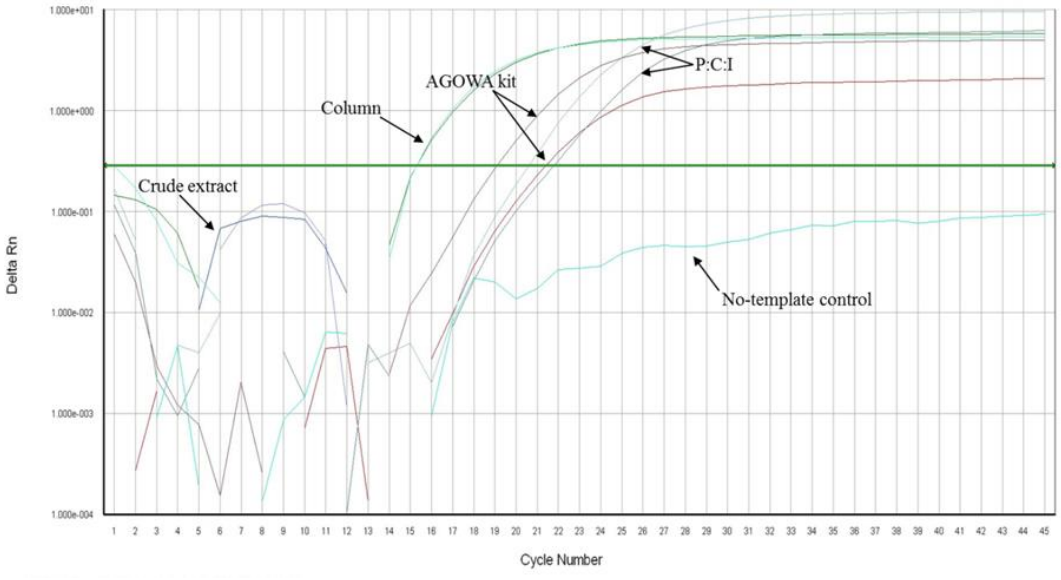


Figure 3.4: Fluorescence vs. cycle number for different *S. aureus* DNA extraction methods

Table 3.4: C_t values for different DNA extraction methods for *S.aureus*

Extraction Method	Sample	C_t
AGOWA®	*S. aureus* 1	21.46
	S. aureus 2	19.15
	Negative control	Undetected
Column	*S. aureus* 1	15.28
	S. aureus 2	15.3
	Negative control	Undetected
Crude	*S. aureus* 1	Undetected (too high)
	S. aureus 2	Undetected (too high)
	Negative control	Undetected
P:C:I	*S. aureus* 1	21.92
	S. aureus 2	20.62
	Negative control	Undetected

Gram negative bacteria

DNA from samples containing 2.0 x 10^8 *K. pneumoniae* cells was extracted and processed in duplicate using each of the 4 methods described above. Amplification from the crude DNA extraction occurred earliest at cycle 16 (Figure 3.5). This was followed by the column-based method amplifying at around a cycle number of 17. The replicates for each of the 2 methods amplified at the same time.

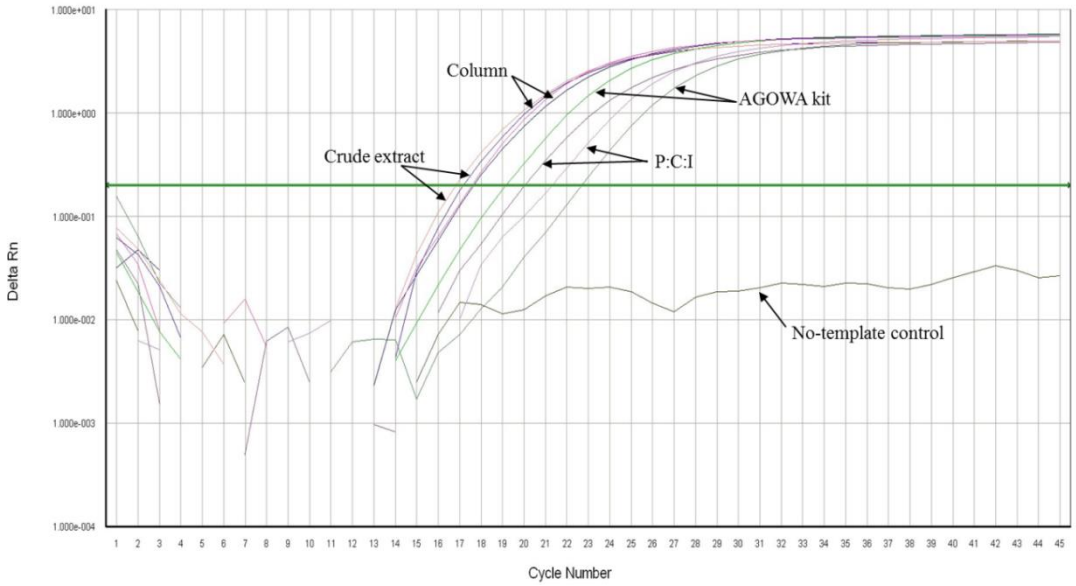


Figure 3.5: Fluorescence vs. cycle number for different *K. pneumoniae* DNA extraction methods.

The AGOWA® and P:C:I methods had amplification at later cycles (between 18 and 22) with a difference in 3 cycles between replicates for the AGOWA® method (Table 3.5).

Table 3.5: C_t results for different DNA extraction methods for *K. pneumoniae*.

Extraction Method	Sample	C_t
AGOWA®	*K. pneumoniae* 1	18.8
	K. pneumoniae 2	22.33
	Negative Control	Undetected
Column	*K. pneumoniae* 1	17.24
	K. pneumoniae 2	17.3
	Negative Control	Undetected
Crude	*K. pneumoniae* 1	16.86
	K. pneumoniae 2	16.46
	Negative Control	Undetected
P:C:I	*K. pneumoniae* 1	19.68
	K. pneumoniae 2	20.99
	Negative control	Undetected

3.2.2.3 Comparison of DNA extraction methods discussion

The modified Qiagen column-based DNA extraction protocol appeared to be the best method of those tested. Amplification of DNA extracted using the columns occurred earlier than other methods for *S. aureus* and the second earliest for *K. pneumoniae*. The crude method would be unsuitable for many downstream techniques due to the presence of inhibitors which would not be removed. Column based methods are widely used and are capable of removing a large proportion of compounds that may inhibit PCR through binding of nucleic acid to the silica membrane and optimisation of buffers to neutralise inhibitors (225). Phenol and chloroform-based methods can have the risk of chemical carry-over if care is not taken, which could interfere with PCR amplification. Also, the method does not lend itself well to higher throughput

sample processing, along with the AGOWA® method, which has the disadvantage of only being able to process 6 samples at once on a magnetic strip. Moreover, the latter two methods did not produce accurate replicate data and could therefore not be relied upon to produce consistent results in the study.

3.2.3 Optimisation of DNA extraction method for swab samples

In order to test the suitability of the chosen DNA extraction method for the project, test swab samples were taken from the hospital to be sampled in the study. DNA extractions were performed using the Qiagen column method as described above and end-point PCR was carried out to determine if nucleic acid was present and if the method was sensitive enough for the sample type.

3.2.3.1 Optimisation of DNA extraction for swab samples method

Swabs were taken at each of the following sites:

1. Chair arm
2. Bed rail
3. Floor
4. Sink
5. Windowsill
6. Bin
7. Table
8. Chair arm
9. Top of alcohol dispenser
10. Top of drug cabinet
11. Entertainment monitor
12. Air sample on gelatine filter

Swabs were taken as described in Section 2.2.2.1. The swabs in AE buffer were vortexed for 30 seconds and 200 µl was taken to be used for DNA extraction using

the Qiagen column method as described in Section 3.2.2.1. End-point PCR was carried out on the samples using 16S rDNA primers 8A, 8B and 3R as described in Table 2.3. The PCR products were then analysed by gel electrophoresis (Section 2.7.1).

3.2.3.2 Optimisation of DNA extraction for swab samples results

All samples failed to show a band on a gel, indicating the absence of DNA.

3.2.3.3 Optimisation of DNA extraction for swab samples discussion

The failure of the above experiment could have been due to a number of reasons. The DNA extraction may have failed for this sample type, the PCR conditions may not have been optimum, inhibitors may have been present, or there may have been insufficient template added to the reaction. The Molzyme brand of *Taq* polymerase, used for this assay, is reported to lose activity rapidly and also to vary between batches (Kathryn Harris, personal communication 2012) and could be a reason for lack of amplification.

3.2.4 Comparison of polymerase brands for DNA amplification and testing for inhibition

The Molzyme brand of *Taq* polymerase, MolTaq, has been found to lose activity over time. An alternative *Taq* polymerase: BIOTAQ™ (Bioline, UK), was tested with

the same samples as before and *E. coli* DNA was added to samples as an IPC to check for inhibition.

3.2.4.1 Comparison of polymerase brands for DNA amplification and testing for inhibition method

End-point 16S rRNA gene PCR was carried out on the same test swab extracts as before (Section 3.2.3.1) with double the amount of MolTaq added. PCR was also carried out on the same samples using BIOTAQ™ *Taq* polymerase in place of MolTaq. Four samples had 1 µl *E. coli* genomic DNA IPC added and were included in the PCR run with each polymerase.

3.2.4.2 Comparison of polymerase brands for DNA amplification and testing for inhibition results

Amplification only occurred with the use of double concentration MolTaq polymerase. Figure 3.6 shows the gel image of PCR products. Lane 2 of the top row (MolTaq) shows the sample from a bin (Sample 6) and was very weakly positive. Lanes 7- 10 contained spiked *E. coli* DNA and a positive result occurred in 3 out of 4 samples. No amplification occurred when BIOTAQ™ was used.

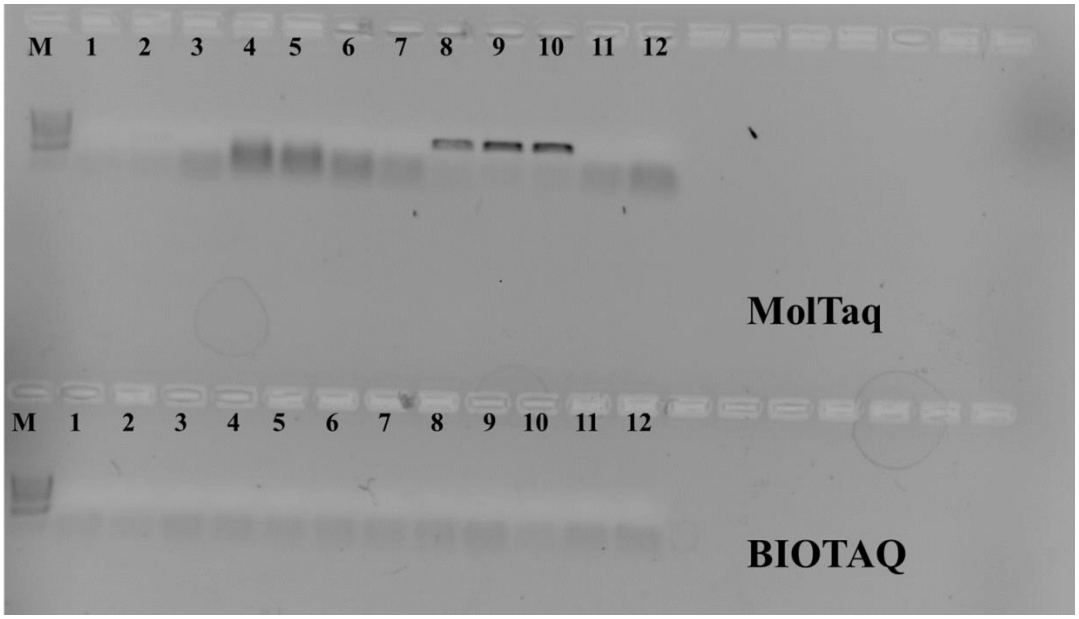


Figure 3.6: Gel electrophoresis image of 16S rDNA PCR products comparing *Taq* polymerases. Top line MolTaq, bottom line BIOTAQ

3.2.4.3 Comparison of polymerase brands for DNA amplification and testing for inhibition discussion

Amplification was only observed in one sample when using double the recommended concentration of MolTaq polymerase. The use of BIOTAQ™ yielded no positive results. The IPC was positive for 3 of 4 samples when using MolTaq, indicating that PCR conditions were sufficient for DNA amplification and that inhibition was not the reason for failure to amplify, at least in 3 samples. The lack of any amplification when using the BIOTAQ™ could be explained by sub-optimal PCR conditions. Given that IPC amplification occurred with MolTaq, this indicated that the samples may have been the problem, perhaps due to low DNA concentration and not necessarily the type of polymerase. Although, the use of double the

recommended concentration of MolTaq indicated a loss of enzyme activity and a new batch was acquired.

3.2.5 Repeat testing of swabbing, DNA extraction and PCR methods

A new set of swabs were taken as before and the DNA extraction and PCR repeated as before but with new reagents.

3.2.5.1 Repeat testing of swabbing, DNA extraction and PCR method

Swabs were taken, DNA extracted using the modified Qiagen method and end-point PCR carried out using the 8a/8b and 3F primers, all as before. All swabs samples also had 1 μl IPC DNA added in a separate PCR reaction.

3.2.5.2 Repeat testing of swabbing, DNA extraction and PCR results and discussion

The gel image is shown in Figure 3.7. Lanes 1 – 11 show swab samples, lane 12 shows an air sample and lane 13 shows a blank swab. Lanes 14 – 26 show samples with IPC added. Lanes 27 and 28 are NTCs and positive controls are in lanes 29 and 30. The marker has been cut off the picture; however the bands were the expected size.

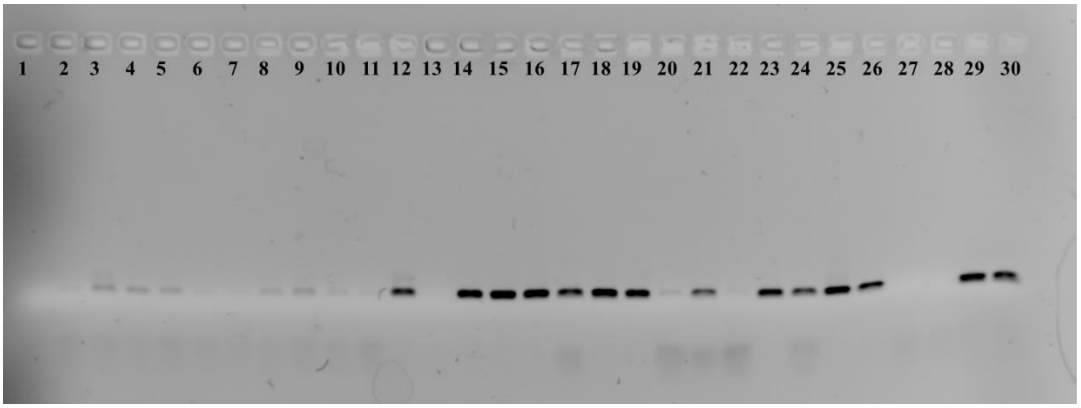


Figure 3.7: Gel electrophoresis picture showing amplified DNA from test swabs.

Lane 1: Chair arm, 2: Window ledge, 3: Floor, 4: Sink, 5: Bed rail, 6: Alcohol gel dispenser, 7: Drug cabinet, 8: Bin, 9: Entertainment monitor, 10: Patient table, 11: Light, 12: Air sample, 13: Blank swab, 14 – 26 as before with 1 μl *E.coli* DNA, 27 & 28: NTC, 29 & 30: Positive control.

The majority of the swab samples were positive but in comparison to positive controls and samples with IPC, had much weaker bands (Figure 3.7), indicating the presence of less starting template DNA. The drug cabinet (Lane 7) and entertainment monitor (Lane 9) did not amplify well and the IPCs for these samples only showed weak amplification (Lanes 20 and 22), indicating the presence of inhibitors. Some samples that were expected to have large quantities of DNA present, such as the chair arm did not amplify. However, the air sample showed good amplification. NTCs did not amplify and positive controls had amplification. The extraction method appeared to work for the sample type; however, DNA yield was low regardless of swab site.

3.2.6 Comparison of methods to increase DNA yield from swabs

The DNA yield from swabs was low in the above experiment. In order to attempt to improve DNA yield from environmental swabs, the DNA extraction method used in the above experiments was further modified.

3.2.6.1 Comparison of methods to increase DNA yield from swabs method

Further test swabs were taken from the hospital: 1) Floor, 2) Table, 3) Light, 4) Sink, 5) Chair arm, 6) Bed rail and 7) Air. A set of swabs was extracted using each of the methods given below. End-point 16S rRNA gene PCR was carried out as before and 16S rRNA gene qPCR was also carried out as it is a more sensitive method for DNA detection (Table 2.9). IPC DNA was added to each set for end-point PCR. Air samples were only processed with Method A.

Method A: Increased proteinase K incubation time.

The Qiagen column method as described in Section 3.2.2.1 with proteinase K incubation time extended to 1 hour from 10 minutes.

Method B: QIAshredder column with increased proteinase K incubation.

Swab lysate and swab tips were placed in a QIAshredder column (Qiagen, UK) and centrifuged for 5 minutes at 16,300 x g (13,300 rpm). The eluate was then extracted using the method given in Section 3.2.2.1, with 1 hour proteinase K incubation.

Method C: QIAamp column method

Qiagen column extraction method as described per Section 3.2.2.1. With a 10 minute proteinase K incubation.

Method D: No bead beat, QIAshredder column with increased proteinase K incubation

Method as before (Section 3.2.2.1) but without the initial bead-beat of samples and with 1 hour proteinase K incubation.

In order to check for inhibition, PCRs were repeated, with samples from methods A and B at a 1: 10 dilution and at a 1: 10 dilution with IPC added.

3.2.6.2 Comparison of methods to increase DNA yield from swabs results

When analysing by qPCR, all samples amplified earlier with method D than the other methods (Table 3.6). Samples processed with method C, the original method,

amplified latest overall. Samples that were expected to have less DNA present, such as the bed rail, amplified at later cycles than those expected to have more DNA, such as the light, when using method D. This trend was not as pronounced for the other methods and the bed rail sample amplified before the light when using method B and at the same time as the floor and light when using method C (Table 3.6). NTCs run on this assay had C_t values of around 22, similar to many of the samples, particularly for method C.

The end-point PCR results indicated that methods A and B were better for all sample types (Table 3.7). However, some IPCs failed to amplify for all methods.

Table 3.6: Average C_t values for different methods of DNA extraction from test swabs

	Average C_t value			
	Method			
Sample	**A**	**B**	**C**	**D**
Floor	19.88	19.07	22.81	19.17
Table	21.96	19	22.96	18.96
Light	19.48	22.47	22.22	17.85
Sink	20.13	21.12	21.37	19.17
Chair arm	20.29	19.06	20.33	18.88
Bed rail	21.38	21.58	22.19	20.31
Air	19.99	N/A	N/A	N/A

Table 3.7: Comparison of DNA extraction methods by end-point 16S rDNA PCR

	Method			
Sample	**A**	**B**	**C**	**D**
Floor	+	+	-	+
Table	-	+	-	+
Light	+	-	-	-
Sink	+	+	-	-
Chair arm	+	+	-	-
Bed rail	-	-	-	-
Air	+	N/A	-	N/A

Due to the lack of IPC amplification in some samples, those samples were all diluted to 1:10 and PCR was repeated as normal and with IPC added.

Samples diluted to 1:10 showed very little or no amplification but IPCs, which had previously all been negative. The samples with IPC showed a lot of smearing and some non-specific bands (Figure 3.8).

3.2.6.3 Comparison of methods to increase DNA yield from swabs discussion

Method D appeared to be the best extraction method when using qPCR to amplify 16S rDNA. The DNA from most samples amplified earlier than with other methods, indicating the presence of more DNA in the sample. The QIAShredder used in methods B and D is designed to break up swabs in order to release more cells for exposure to the DNA extraction method.

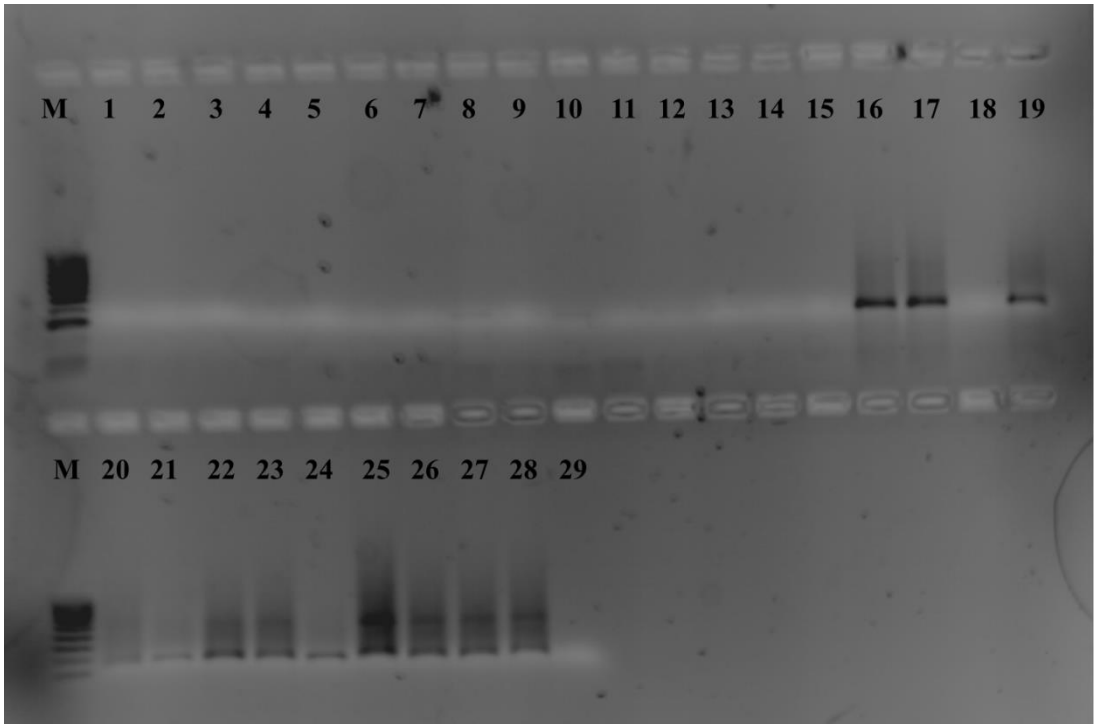


Figure 3.8: 1:10 dilutions of samples extracted with methods A& B and 1:10 samples spiked with IPC DNA. Lanes 1, 15 & 29: NTCs. Lanes 2 – 8: 1:10 dilutions of samples extracted with method A, lanes 9–15: 1:10 dilutions of samples extracted with method B. Lanes 16 – 28: samples as before at a 1:10 dilution with 1 µl IPC DNA spiked into the reaction.

Methods A and B gave best results when using end-point 16S rRNA gene PCR. Both of these methods used an extended proteinase K step and this may allow more DNA to be freed from protein associations which may prevent amplification. Diluting samples to 1:10 did not produce positive results from samples that were previously negative but expected to have sufficient DNA present. However, in some cases, diluting did lead to the amplification of IPC DNA, indicating that inhibition might be causing false negatives. The above experiments indicated that the QIAshredder did not provide a large improvement in DNA yield but that extended incubation with

proteinase K did. The experiments also indicated that further PCR optimisation was required to provide better results from swab samples.

3.3 PCR optimisation for the detection of DNA from environmental swab samples

During the method development so far discussed, 16S rRNA gene end-point PCR was carried out using primers 8A, 8B and 3R. Other primer sets are available and at this stage in project development, primer choice for next-generation sequencing was considered. Primer sets 8A, 8B and 3R ('GOSH' primers) (141), which target the V1-V3 hypervariable regions of the 16S rRNA gene, were tested along with 785F and 1175R ('Eastman' primers), which target the V5-V7 regions (214) and a set developed by Caporaso et al. for NGS on environmental samples, targeting the V4 region (145). PCR cycling conditions were altered along with types of *Taq*. The experiments are outlined below.

3.3.1 GOSH *vs.* Caporaso primers with DNA from pure bacterial cultures

GOSH primers had previously been used in order to test DNA extraction methods as these were in use routinely at GOSH and this aided troubleshooting. However, at the time of conducting the study and of writing, the V4 region is the most common region amplified when investigating microbial communities (71,145,226). Therefore, a primer set to amplify this region was obtained from the literature (145). In order to

test ‘best-case scenario’ PCR amplification, a dilution series from 1 X 10^{12} to 1 X 10^{1} of *K. pneumoniae* DNA was amplified with Caporaso and GOSH primers.

3.3.1.1 Method

A ten-fold DNA dilution series was prepared by diluting DNA in AE buffer. PCR reagents and cycling conditions are detailed in Table 2.3 for GOSH primers. Caporaso primers were used with the same parameters.

3.3.1.2 GOSH *vs.* Caporaso primers with DNA from pure bacterial cultures results

DNA dilutions from 10^{12} to 10^{5} could be visualised on an agarose gel when GOSH primers were used as shown in the top row of Figure 3.9. Only the highest 3 dilutions amplified when using Caporaso primers and the 10^{10} was only very weakly positive.

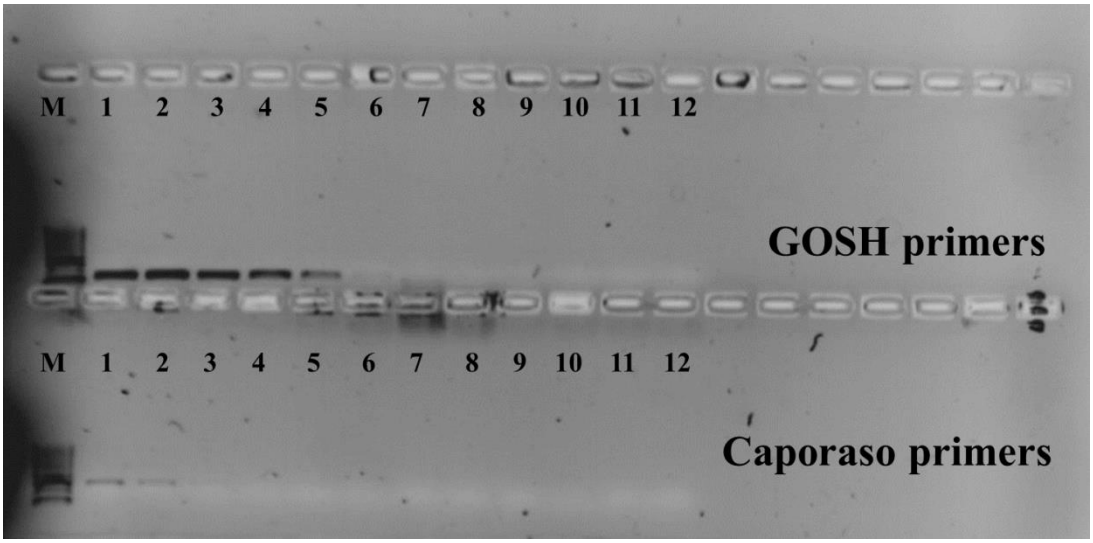


Figure 3.9: Dilution series of *K. pneumoniae* DNA amplified using GOSH & Caporaso primers. Top and bottom rows: 1) 10^{12}, 2) 10^{11}, 3) 10^{10}, 4) 10^{9}, 5) 10^{8}, 6) 10^{7}, 7) 10^{6}, 8) 10^{5}, 9) 10^{4}, 10) 10^{3}, 11) 10^{2}, 12) AE control

3.3.2 GOSH *vs.* Eastman *vs.* Caporaso primers on DNA from swab extracts

A third primer set; 785F and 1175R (Eastman primers) was added to those to be tested as they amplify the V5-V7 hypervariable regions of the 16S rRNA gene. The V6 region, in particular, is known to be highly discriminatory in bacterial identification (138,147). The 3 primer sets were tested using test swabs from the hospital.

3.3.2.1 GOSH *vs.* Eastman *vs.* Caporaso primers on DNA from swab extracts method

Test swabs were taken from 2 of each of the following items on the ward: chair arm, floor, bed rail, sink and bin. These were extracted and as before and PCRs carried out using the conditions described in Tables 2.3 and 2.4. Extraction controls in the form of un-used swabs, NTCs and positive controls were included.

3.3.2.2 GOSH *vs.* Eastman *vs.* Caporaso primers on DNA from swab extracts results

No amplification occurred except for the air sample with both GOSH and Eastman primers. The use of alternative PCR conditions was therefore considered.

3.3.3 Use of a proof-reading polymerase for improved PCR

MolTaq polymerase batches vary considerably in their activity and this may have been a contributing factor to inconsistent results. KAPA Hi-Fi *Taq* polymerase (KAPA Biosystems, UK) was tested due to its reported ability to amplify low concentration DNA and increase yield. It is a proof-reading *Taq* polymerase and as such is thought to produce less chimeric DNA amplicons (227).

3.3.3.1 Use of a proof-reading polymerase for improved PCR method

Swab extracts were used as before and DNA amplified with the GOSH primer set using KAPA *Taq* in place of MolTaq. Cycling conditions were as follows; 1 initial denaturation at 95 ^{O}C for 5 minutes, followed by 35 cycles of 98 ^{O}C for 20 seconds, 60 ^{O}C for 30 seconds, 72 ^{O}C for 1 minute and a final elongation at 72 ^{O}C for 5 minutes.

3.3.1.2 Use of a proof-reading polymerase for improved PCR results

All samples showed excessive non-specific bands on an agarose gel, as did the extraction controls and NTC (Figure 3.10). Therefore, the KAPA *Taq* was not tested further and other PCR conditions were altered to attempt to improve results.

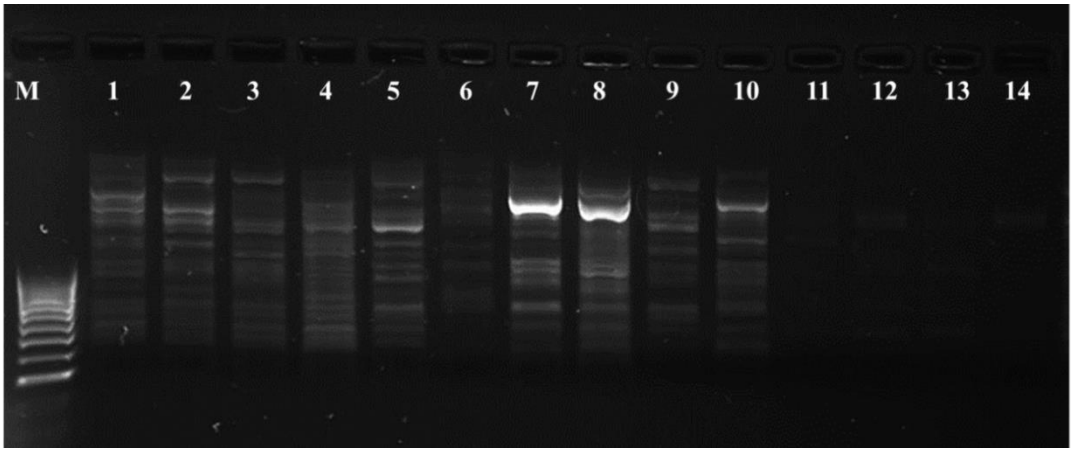


Figure 3.10: PCR amplification with KAPA *Taq* of test swabs with GOSH primer set M: Hyperladder, Lanes 1 & 2: chair arm, 3 & 4: floor, 5 & 6: bedrail, 7 & 8: sink, 9 & 10: bin, 11 & 12: extraction control. 13: NTC, 14: positive control

3.3.4 Eastman primer PCR with DNA from swab extracts and altered PCR conditions

Test swabs, as before, were amplified using the Eastman primer set, MolTaq polymerase and altering the PCR annealing temperature to 60 ^{O}C.

3.3.4.1 Eastman primer PCR with DNA from swab extracts and altered PCR conditions results

Bands were observed for all swab samples, except the bed rails (Figure 3.11). The NTC was negative.

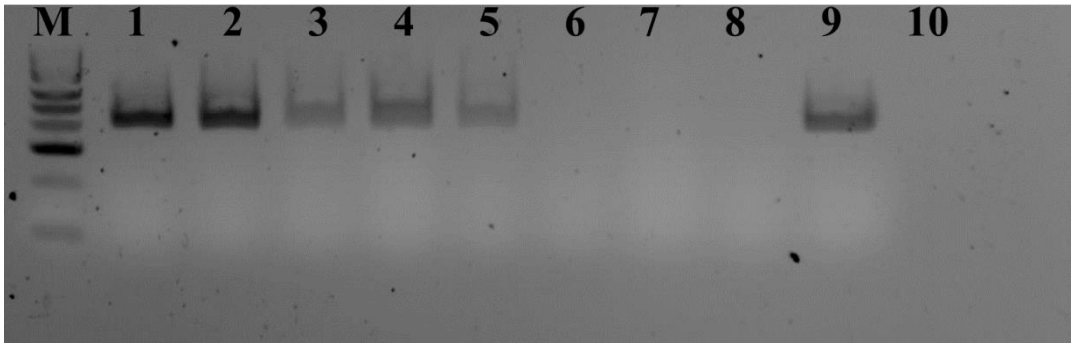


Figure 3.11: Test swabs amplified with MolTaq and higher annealing temperature. M: Hyperladder, 1 & 2: chair arm, 3 & 4: floor, 5 & 6: sink, 7 & 8: bed rail, 9: positive control, 10: NTC

3.3.4.1 Eastman primer PCR with DNA from swab extracts and altered PCR conditions discussion

Altering the annealing temperature of the PCR reaction appeared to result in improved DNA amplification. Bed rail samples still failed to amplify but these samples were expected to have lower numbers of bacteria present and this may explain the lack of amplification.

3.4 Method development for MiSeq NGS

Due to the significant cost involved with NGS, it was not possible to conduct 'trial runs' of sequencing in order to optimise methods. The Illumina MiSeq was chosen for this project due to the low relative cost and long read-length in comparison to other available technologies. A review of NGS platforms is given in Chapter 6. MiSeq protocols were designed using available literature, Illumina methods and advice from colleagues using the technology and developed throughout the project.

For clarity, the final method used is presented in Chapters 2 and 6 but challenges and method development for the MiSeq are discussed below. Results of successful sequencing runs are presented in Chapter 6.

The workflow for the NGS of DNA from swab samples is shown below (Figure 3.12), this is also discussed in further detail in Chapter 6 but an overview is provided here to aid the method development information to be better understood. DNA is extracted and amplified by PCR to add barcode sequences which allow sequenced amplicons to be traced back to their original sample. PCR products are purified, adjusted to equimolar concentrations and pooled to form a library. The library is quantified and chemically denatured with sodium hydroxide (NaOH). The library is then bound to a flow cell which is a glass slide with oligonucleotides bound where PCR occurs to amplify the library. The amplified library is then sequenced.

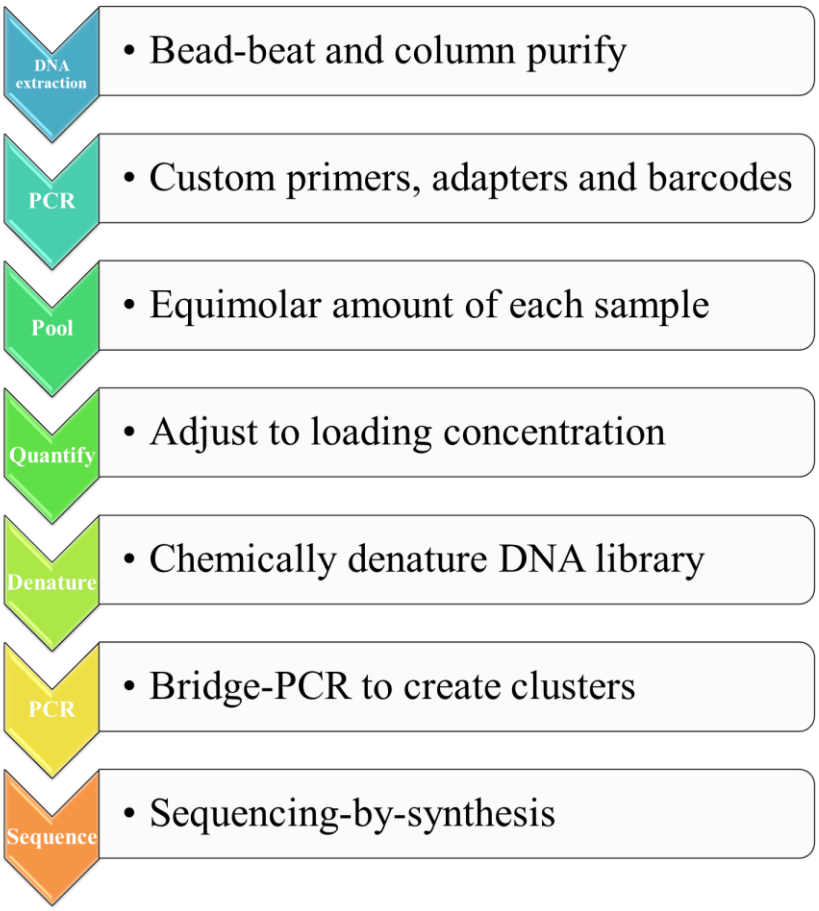


Figure 3.12: Workflow for sample preparation for next-generation sequencing.

3.4.1 Initial MiSeq sequencing run method

Barcode PCRs were carried out on 96 pooled DNA samples from the ward as detailed in Section 2.6.1.4 (details of pooling given in Section 2.10.2.1). PCR products were purified on Size Select® gels and samples were recovered and quantified using the Qubit® (Section 2.9.2.1). Only 53 of a potential 96 samples were carried forward as the remainder did not amplify to a high enough concentration. The sequencing protocol, as detailed in Sections 2.10.2.2 – 2.10.2.6 was carried out.

3.4.1.1 Results of initial MiSeq run

The samples failed to cluster on the Illumina flow cell after starting the MiSeq run and no data were recovered.

3.4.1.2 Discussion of initial MiSeq Run

After extensive research, a reason for run failure could not be determined. It is known that residual NaOH from the library denaturation stages may prevent samples binding to the flow cell and the presence of traces of other chemicals has not been investigated. The presence of residual chemicals from the swab samples may have been a reason for the samples failing to bind to the flow cell. Therefore, all samples which showed inhibitory effects by IPC qPCR assay (Section 2.6.2.7) were removed from their respective pools, the samples re-pooled and re-processed.

3.4.2 Optimisation of protocol for further MiSeq runs

Barcode PCRs were carried out as before on fresh pools of samples but failed to amplify well, possibly due to lower concentrations of DNA present in the pool. The protocol and workflow were adjusted to compensate for the lower concentrations of DNA. PCRs were repeated, adding more template sequentially in order to obtain amplification and the reaction volume was increased to 50 µl by doubling the amounts of each reaction component.

Some samples showed positive results but others still failed to amplify to a concentration that could be recovered from a Size Select® gel. Despite bands initially being visible on a gel, when they reached the collection well, bands were no longer present. The purification step, as carried out by Size Select® gel, is to ensure the accurate loading of DNA library onto the MiSeq flow cell. The Qubit® method quantifies all dsDNA, including primer-dimers, which if not removed by gel purification, will lead to an underestimate of DNA concentration. This will cause further steps in the sequencing process to be inaccurate and may lead to run failure. Alternative purification methods were tested to attempt the recovery of lower concentration samples.

3.4.3 Alternative DNA purification methods

GE illustra Sephadex™ columns as described in Section 2.8.2 were tested against a bead-based purification method using Agencourt® AmPure® XP magnetic beads (Beckman Coulter, UK). Two sample pools which had shown previous positive bands on a gel were used to test the purification methods. Samples A and B were amplified in quadruplicate using barcode PCR and quantified. Duplicates of each sample were purified using Sephadex™ columns or using AmPure® beads (protocol below). An AE buffer NTC was included for each method.

3.4.3.1. AmPure® XP Magnetic Bead method

AmPure® XP magnetic beads were equilibrated to room temperature and vortexed for 30 seconds to resuspend. 1.8 µl of AmPure® XP beads per 1 µl PCR product were added to the well of a conical-bottom 96 well plate (ABI, UK). In this case, 49 µl was used (1 µl was used to quantify); therefore 88.2 µl beads were added.

PCR product and beads were mixed thoroughly by gentle pipetting and incubated at room temperature for 5 minutes to allow DNA binding. The plate was then placed on an Agencourt® Solid Phase Reversible Immobilisation (SPRI) 96 Super Magnet Plate (Figure 3.13) for 2 minutes to allow the beads to cluster to one side of each well. The supernatant was removed and discarded.

The beads were washed twice with 70 % ethanol to remove contaminants whilst remaining on the magnetic plate. The beads were then gently rinsed from the side of the well with 40 µl PCR-grade water (elution fluid), mixed well, incubated for 2 minutes at room temperature then placed back on the magnetic plate. The supernatant was removed and stored at 4 ^{O}C until required.

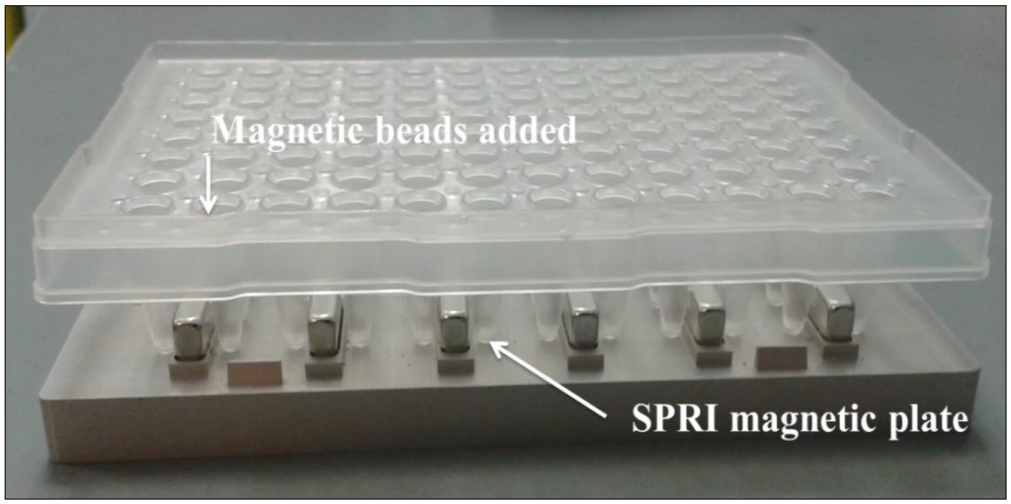


Figure 3.13: Solid Phase Reversible Immobilisation magnetic plate with conical-bottom 96 well plate

3.4.3.2 Results of alternative DNA purification methods

Table 3.6 shows the DNA concentration (ng / µl) prior to PCR purification and then after purification by the Sephadex™ column or magnetic bead method. It can be seen that after PCR, the samples were of similar concentration and the NTC had very little DNA present. Both methods of purification reduced the DNA concentration to very low levels, on one occasion the bead method reduced the DNA to undetectable levels (Sample A). Concentration in nM is also shown, Illumina recommend a starting concentration of 4 nM or 2 nM in their MiSeq sample preparation protocol (www.illumina.com) and no sample reached that level.

Table 3.6: DNA concentration before and after different purification methods

		DNA concentration (ng / μl)		DNA concentration (nM)
Sample	**Clean-up**	**PCR product**	**After purification**	**After purification**
A	Sephadex™	2.64	0.144	0.4
		2.32	0.26	0.79
	AmPure®	2.9	Too low	-
		2.78	0.14	0.44
B	Sephadex™	2.96	0.144	0.46
		3.06	0.216	0.69
	AmPure®	2.66	0.206	0.65
		2.94	0.232	0.74
AE	Sephadex™	0.242	Too low	-
	AmPure®	0.304	Too low	-

In order to compare DNA loss between the two above methods and the Size Select® gel method, the same samples were repeated using the barcode PCR and run on a gel. The bands were very faint and after recovery, all were too low for detection on the Qubit®. None of these methods would allow library preparation for NGS to be achieved.

3.4.3.3 Testing higher concentration PCR products

In order to identify whether the purification methods were appropriate when higher concentrations of PCR product were used, two further samples which had previously amplified well were then compared using the methods above (samples C and D). Results are shown in Table 3.7. Despite sample C having similar starting DNA concentrations to samples A and B, recovery after purification was better in this

instance. However, recovery of both samples using the AmPure® beads was much lower than when using Sephadex™ columns.

Table 3.7: DNA concentration before and after purification methods using higher starting concentrations.

		DNA concentration (ng / µl)		DNA concentration (nM)
Sample	**Clean-up**	**PCR product**	**After purification**	**After purification**
C	Sephadex™	1.97	0.666	2
	AmPure®	1.92	0.108	0.33
D	Sephadex™	4.12	1.43	4.4
	AmPure®	4.46	0.492	1.51

Sephadex™ purified sample D was then run on a Size Select® gel to check for primer dimers and other non-specific bands. After recovery from the gel, DNA concentration was recorded at 1.4 nM, meaning that approximately ¾ of the starting DNA was lost in the gel. A secondary band was observed when running the gel which was smaller than the amplicon size and this was assumed to be primer-dimer and accounted for some of the loss.

3.4.3.4 Purification comparison discussion

Based on the above experiments, neither the Sephadex™ columns nor AmPure® bead method appeared appropriate for use when beginning with low concentration PCR products, the loss was too great. Higher DNA concentrations post-PCR offered sufficient DNA recovery for the Illumina library preparation protocol when using the Sephadex™ columns. However, these columns do not allow volumes of PCR product

added to be adjusted, meaning that they could not be concentrated in order to achieve the highest possible yield. Also, the matrix size used in these columns may allow DNA fragments of 50 bp to be co-purified. Given that the primers used in the barcode PCR are 62 and 68 bp long, there is potential for primer dimers to also be purified when using this method, which would lead to incorrect library quantification.

Bands smaller than the 16S rDNA indexed amplicon were observed when running sample D (and other later samples) on a Size Select® gel after Sephadex™ purification, indicating that purification using this method was not complete and it could not be used alone when preparing a DNA library. Concentrations of DNA recovered were lower when using the AmPure® beads. This method also has the potential to carry over smaller DNA fragments and is not well suited to high throughput applications. It was therefore determined that a Size Select® gel must still be run prior to library pooling and that alternative purification methods would not provide an advantage. However, Size Select® gels had previously been shown to not be suitable for purification of low concentration PCR products and this required further investigation.

3.4.4 Recovery from Size Select E-gels®

To attempt to improve recovery of PCR products when using Size select® gels, barcode PCR using 10 µl of template was conducted as before on 9 pooled samples. The PCR products were quantified and then run on a Size Select® gel. The results

showing starting DNA concentration and concentration after gel purification are presented in Table 3.8.

It can be seen that over half of the samples run on the gel did not have enough DNA recovered for the NGS protocol (E – K & N). The remainder of the samples were at a high enough concentration (> 1nM) after purification to construct a MiSeq DNA library.

Table 3.8: DNA concentration of PCR product before and after Size Select E-gel® purification

	DNA concentration (ng / μl)		DNA concentration (nM)
Sample	**PCR product**	**After gel**	**After gel**
E	1.63	Too low	Too low
F	1.79	Too low	Too low
G	1.81	0.1	0.32
H	2.32	0.16	0.51
I	2.28	0.21	0.67
J	1.88	0.22	0.7
K	1.98	Too low	Too low
L	4.36	1.17	3.59
M	5.06	0.72	2.3
N	13.5	0.28	0.89
O	13.9	1.5	4.77
P	12.8	1.23	3.91
Q	13.4	1.13	3.59
R	14.5	1.73	5.5
S	10.9	1.91	6.07

3.4.4.1 Gel recovery discussion

The above experiment showed that if the concentration of DNA after PCR amplification was above 4 ng / µl, enough DNA was present to run through a Size Select® gel and recover for MiSeq NGS. Lower concentration PCR products were lost when using Size Select® gels.

3.4.5 Purification method for low DNA concentration samples.

Low concentration barcode PCR products had thus far been unable to be purified whilst retaining a concentration appropriate for MiSeq NGS library construction. For reasons previously mentioned, samples must be purified before MiSeq NGS, so an alternative was sought.

Sephadex™ columns do not allow the concentration of DNA by alteration of the elution buffer volume. It was considered that if the concentration of PCR products was low, they could be concentrated using a QIAquick PCR clean-up column, in which DNA is bound to a membrane and can be eluted in a smaller volume (30 µl). The method, outlined in Section 2.8.1, was used to purify PCR products and the concentration was determined, as before, prior to and after purification. The results are shown in Table 3.9.

Table 3.9: DNA concentration before and after QIAquick column purification

	DNA concentration (ng / μl)		DNA concentration (nM)
Sample	**PCR product**	**After QIAquick**	**After QIAquick**
T	1.74	0.29	0.92
U	1.93	0.28	0.89
V	2.53	0.76	2.41
W	1.55	0.25	0.79
X	3.02	0.68	2.16
Y	3.6	0.89	2.83
Z	2.06	0.32	1.02

Here, again it can be seen that for higher starting concentration PCR products, as in the case of samples V, X, Y and Z, recovery was high enough for NGS library preparation (> 1nM). However, given that the previous non-gel based purification methods had failed to adequately 'clean-up' the samples, the higher concentration samples were run on a Size Select® gel to check for non-specific bands (Figure 3.14).

Samples V, X, Y and Z produced small bands on a Size Select® gel but also a large band between the 50 and 100 bp marks. This indicates the presence of unincorporated primers and possible primer-dimers, further highlighting the requirement to purify samples on a gel prior to NGS.

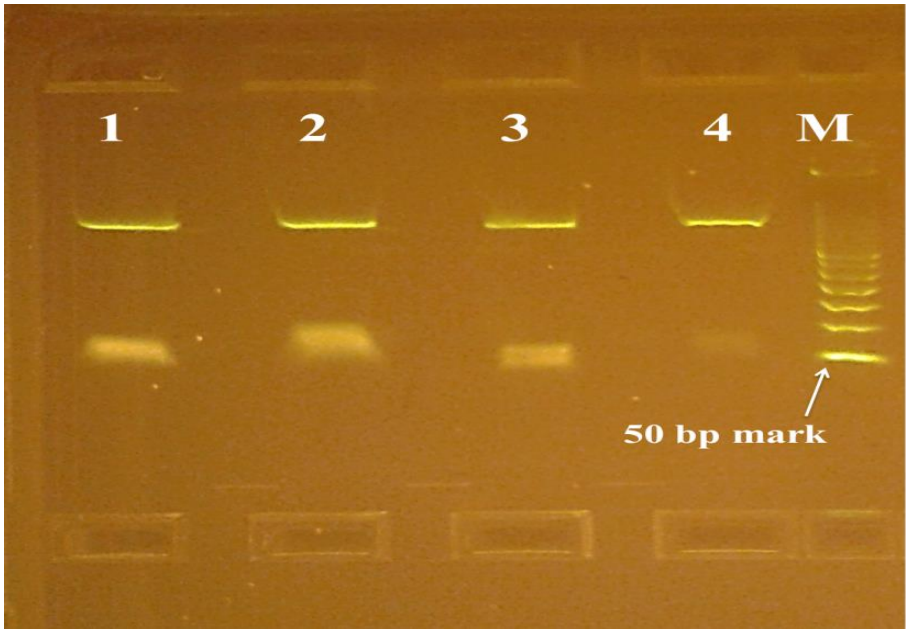


Figure 3.14: DNA samples after QIAquick purification run on Size Select E-gel®.

A high proportion of samples still had a DNA concentration after PCR that was too low to purify using any of the methods tested thus far. Excluding these samples would have resulted in a large loss of data for the study presented in Chapter 6. Therefore, it was decided to carry out barcode PCR on each of these samples and quantify them post-PCR. Each sample would then be adjusted to an equivalent concentration (4 nM) prior to purification. These samples would then be pooled and purified as a single sample on a QIAquick column, eluting in 30 µl of PCR-grade water, thereby concentrating all of the samples. The purified product would then be run on a gel to remove non-specific DNA and quantified.

3.4.6 Discussion and conclusions

The method validation and testing for the project was extensive. The choices made were based on existing knowledge, standard operating procedures and published protocols. They were altered and tested in a logical step-wise process in order to ensure that the final methods chosen would enable any data collected to be the best quality and provide the most information for the study.

3.4.6.1 Environmental sampling methods and PCR development

Of the two types of swab tested, neither proved to perform better than the other when recovering viable bacteria nor free DNA from tile surfaces. Cotton swabs had the advantage as they can be more easily used in the field.

16S rRNA gene PCR worked well for amplifying pure bacterial DNA but with field samples, proved to be problematic. The 16S rRNA gene qPCR did not provide useful information as everything, including the NTCs amplified. Reagent contamination is common, despite the manufacturer's claims that products are PCR-clean. The level of 'cleanliness' is adequate for specific PCRs in which particular microorganisms are targeted but when using a broad-spectrum assay designed to amplify all bacteria, the reagents appear to not be as 'clean' as reported. This is particularly apparent in 16S rRNA gene qPCR which is more sensitive than end-point PCR and shows amplification of reagent contamination that may not be observed in end-point reactions.

Due to the reduced sensitivity of end-point PCR compared to qPCR, weakly positive samples may not be visible on agarose gels. The samples used in the method development study were from an environment where various types of inhibitors may be present and this, coupled with low bacterial load proved to make amplification problematic. However, after adjustment of methods, DNA was amplified from all test sample types. From the above experiments, the extension of proteinase K incubation time from 10 minutes to 1 hour appears to be the best of the methods tested at improving DNA yield. This is likely due to the increased digestion of protein in the samples, leading to more DNA being available for the PCR reaction.

It was decided that the best amplification of DNA from swabs taken from the hospital environment occurred when using the 785F and 1175R (Eastman) primer set with higher annealing temperature than previously used. The primers were also considered a better candidate for NGS protocols than the other primer sets tested with these samples.

The Caporaso primer sets were designed for NGS use, with added barcodes and adapter sequences; this may explain their poor performance when used as standard primers. They were also designed for amplification of microorganisms from soil samples, which likely differ in community structure to those found on clinical surfaces.

It is now known that proof-reading *Taq* polymerase, such as KAPA Hi-Fi *Taq*, can contribute to much higher incidences of chimera formation (227) and this appears to have been shown in the above experiment. Standard ultra-clean *Taq* polymerase is suitable for 16S rRNA gene PCR, despite reported issues with batch-to-batch inconsistencies, which can be mitigated by testing of each batch.

Low quantities of DNA were obtained after PCR from test swab extracts and this was considered likely to also be the case with the samples taken throughout the year for the work presented in Chapter 6, making PCR difficult. It was decided to pool samples from the ward into 4 sample types for each time point; bed space areas, non-bed space areas, sinks and floors.

3.4.6.2 Development of methods for MiSeq NGS

The main obstacle in preparing DNA libraries for NGS was the low concentrations of DNA present and the difficultly in purification of this DNA. Reasons for low concentrations after PCR could include the initial overall low numbers of microorganisms present on swabbed surfaces, or the incomplete removal from swabs, as reported in this chapter. Also, the cleaning agents used in UK hospitals include chlorine, which may have an inhibitory effect on PCR but no knowledge is available on how chemicals or other inhibitors may affect NGS. The presence of low levels of microorganisms, coupled with the presence of inhibitors may have made the amplification of DNA difficult and despite pooling bed space samples, some

pools did not amplify or amplify to the concentrations required to follow the Illumina MiSeq protocol.

Optimisation and standardisation of a method for preparing samples for MiSeq library preparation was troublesome. DNA concentration in samples was not uniform and some samples had undetectable levels prior to PCR (Table 3.10).

Table 3.10: DNA concentration in samples prior to amplification by PCR

Sample	DNA concentration (ng / μl)
A	0.294
E	0.21
F	0.194
H	0.602
I	0.208
L	0.35
Q	Too low
Y	Too low
Z	0.586

No pattern was observed regarding starting DNA concentration and the optimum amount of DNA template to use for barcode PCR; quantity to use could not be predicted by starting concentration. This may have been due to a number of factors, including the barcode sequences themselves as some may amplify with greater efficiency than others. Also, the samples were not necessarily uniform. They were pools of other samples and may therefore have contained PCR inhibitors that varied between pools. Inhibitors have been shown to have different effects in different reactions, having no effect in one but complete suppression in another (228) and

could possibly play a role in the difficulty of amplification. The samples also likely contained different communities of bacteria, some of which may amplify with greater efficiency than others. Despite 2 samples having similar starting DNA concentrations, other factors may have affected amplification. Butler et al. have reported that stochastic variation is a "*fundamental physical law of the PCR amplification process when examining low amounts of DNA*". They note that these random effects are reflected in the irreproducibility of results when analysing the same sample (229). Random fluctuations in efficiency of priming has also been reported (230) and the work presented here appears to be in agreement with both of these authors comments. The additional length of the barcode primers likely added to the inefficiency of the reaction. Each barcode PCR was therefore a 'trial and error' process in order to achieve amplification.

NGS, as with other sequencing techniques, requires that PCR products be purified prior to sequencing. This is to remove salts and other unincorporated PCR reaction components to ensure they do not interfere with the sequencing reaction. In the case of the MiSeq NGS protocol, accurate knowledge of the amount of DNA being sequenced is required. This is largely due to the need to use NaOH to denature the DNA and the quantity used depends on the DNA library concentration. If remaining in excess, NaOH will prevent the DNA library from binding to the flow cell and therefore the calculations must be accurate. Post-sequencing analysis also requires knowledge of library concentration and all samples should be pooled at an equivalent concentration to make relative quantification possible.

The use of a Size Select® gel to remove non-target DNA from PCR reactions was the method of choice as it was quick and required less sample handling. However, when running low concentration PCR products on the gels, faint bands became lost and very little DNA was recovered. Therefore a different clean up method was sought for those low concentration samples. Sephadex™ columns and AmPure® beads were tested but the losses were still too high for low concentration samples. They were not used for high concentration samples as these could be purified on a gel. QIAquick columns allowed the concentration of DNA sample by eluting in less volume than initially applied. This was often enough to provide a high enough DNA concentration for a 1 nM MiSeq library. There were still a number of samples that were too low. These samples were pooled together, purified and concentrated by column and quantified.

Through the testing of various methods, a solid environmental sampling strategy and method for sample preparation for MiSeq NGS, including for low concentration samples, was developed. These strategies are implemented in the following chapters.

4. Comparing microbial density and diversity in 3 indoor locations

4.1 Introduction

A study was conducted in order to gain a preliminary understanding of bacterial counts and identities in 3 different indoor environments where children spend time. The purpose of the study was to determine if observed levels of bacteria were high enough to warrant a more in-depth screening protocol and to assess the suitability of different locations for sampling, as an indicator of how to proceed with the project. Introductions to sampling methods and the microbiology of clinical environments are given elsewhere (Chapters 1 and 6) but an overview of educational environments and the importance of sampling is given below.

4.2. Microbes in schools

Along with hospitals, schools are critical social infrastructures in a society therefore, maintaining a healthy environment and reducing disease transmission risk should inarguably be one of the key agendas in school operation. Currently, low rates of microbiological testing in schools and in other public areas can lead to incomplete

information regarding occurrence of disease transmission related to these locations (231). It is important to understand the microbial communities within public areas and in particular, in schools as poor health in children impacts on wider society. Outbreaks of infection can lead to closure of schools meaning parents may take time off work to care for them, thus impacting on the economy (232). Children attending schools are at particular risk of infectious disease as they spend the majority of their day in close proximity to a large number of other people. Infectious diseases are easily spread when large groups of people congregate (233). Another risk factor for children in particular, is a fall in vaccination rates. In London, for example, there has been a much lower uptake of the 5 in 1 (Diptheria, tetanus, pertussis, polio and *Haemophilus influenzae*), measles, mumps and rubella (MMR) and meningitis C vaccinations over recent years (234). There have also been reports of increased risk of contracting disease, particularly tuberculosis (TB), on public transport due to being in prolonged close proximity to others and poor air quality (235), (236). With UK class sizes increasing beyond government guidelines (234), many British children living in poverty (237), increasing use of public transport, particularly in London (238) and changing profiles of infectious disease due to immigration and drug-resistance (239), (240), children are at an ever growing risk of becoming ill in an environment which is intended to be protective and nurturing.

4.3 Aims of the study

The aims of this part of the project were to compare number and identities of bacteria in three locations to determine if different environments had different

microbial profiles. It was also to determine the overall suitability of sampling methods in preparation for the year-long investigation on a hospital ward presented in Chapter 6.

4.4 Materials and methods

4.4.1 Site selection

Three locations were chosen: a four bed neurology ward (Tiger ward) at GOSH, an outpatient area in the same hospital and a classroom for approximately 30 Year 6 school children age 10-11. Table 4.1 and Figure 4.1 show sampling sites for Tiger ward, Table 4.2 and Figure 4.2 show sampling sites for the outpatient area and Table 4.3 and Figure 4.3 show sampling sites for the classroom. Each location was divided into 4 areas, roughly quarters and treated as sections A-D to facilitate sample collection. Samples were taken once at each location at 09.00 on Mondays of consecutive weeks. Contact plates and swabs were taken at 12 sites in each section (a total of 48 per location). Each sample was given a unique identifying code as follows:

- **Prefix:** Contact plate (C), Swab (S)
- **Location codes**: W = Ward S = School O = Outpatient waiting area
- **Month Code:** 'Pil' for pilot in this instance
- **Area of room:** Quadrant A, B, C or D.
- **Sample number:** 1-12

- **Sample Process:** PCR (PCR), Culture (Cul)

Each sample was therefore given an identifier e.g.: SWPilA1Cul

Samples were taken at varying heights, from objects made from different materials and from those differing in use; i.e. high and low hand-touch sites (45), (241). Where possible, equivalents were sampled in each location; chairs, window sills, floors and sinks. As the three locations were used for very different purposes, it was not possible to sample solely equivalent sites.

Table 4.1: Sampling sites on Tiger Ward

Sample Number	Sample Site	Sample Number	Sample Site
A1	Floor under bed	C1	Floor under bed
A2	Monitor shelf	C2	Monitor shelf
A3	Chair	C3	No sample
A4	Trolley top	C4	No sample
A5	Monitor top	C5	Monitor top
A6	Drawer front	C6	Drawer front
A7	Top of light	C7	Top of light
A8	Bed rail	C8	Bed rail
A9	Sink (right)	C9	Trolley top
A10	Window (right)	C10	Window (left)
A11	Sink (left)	C11	Bed pedal
A12	Bed pedal	C12	No sample
B1	Floor under bed	D1	Floor under bed
B2	Monitor shelf	D2	Monitor shelf
B3	Chair	D3	Chair
B4	No sample	D4	No sample
B5	Monitor top	D5	Monitor top
B6	Drawer front	D6	No sample
B7	Top of light	D7	No sample
B8	Bed rail	D8	No sample
B9	Trolley top	D9	Trolley top
B10	Window (left)	D10	Window (right)
B11	Bed pedal	D11	Bed pedal
B12	No sample	D12	No sample

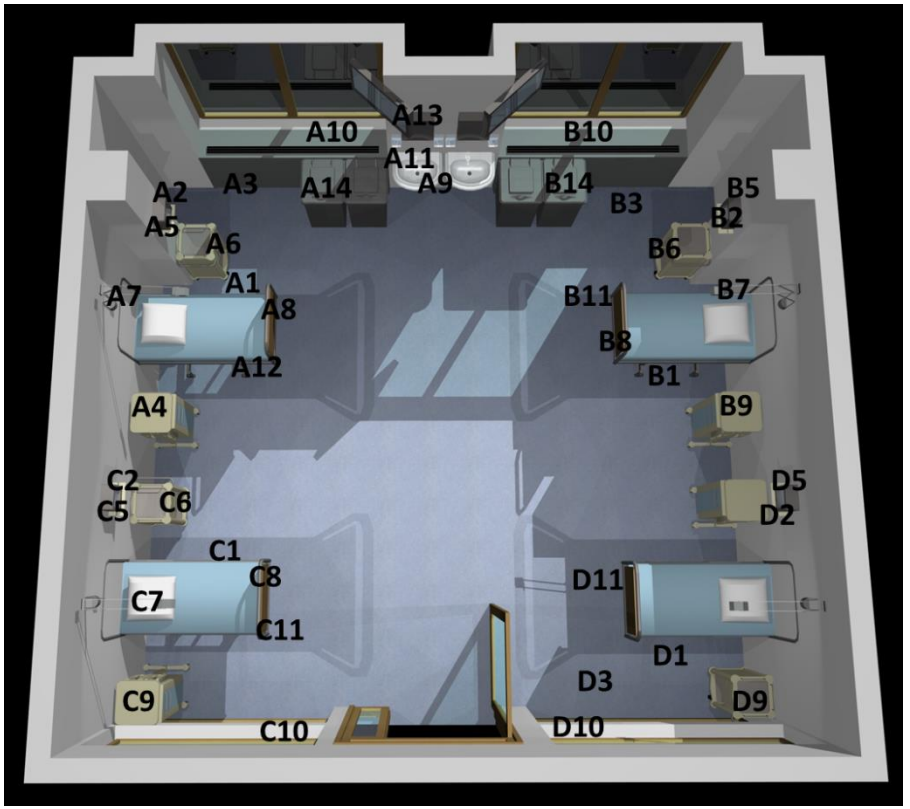


Figure 4.1: Sampling sites on Tiger Ward

Table 4.2: Sampling sites in the outpatient's area

Sample Number	Sample Site	Sample Number	Sample Site
A1	Windowsill	**C1**	Play table
A2	Heating ledge	**C2**	Heating vent
A3	Drawers	**C3**	Toy
A4	Football table	**C4**	Table 2
A5	Main table	**C5**	Chair leg 1
A6	Main table 2	**C6**	Chair leg 2
A7	Top of cupboard 1	**C7**	Door
A8	Front of cupboard 2	**C8**	Handle female WC
A9	Under main table	**C9**	Sign
A10	Chair	**C10**	Chair
A11	Floor under football table	**C11**	Corner trunking
A12	Top of cupboard 2	**C12**	Floor by female WC
B1	Top of cupboard 3	**D1**	Table 1
B2	Main desk 1	**D2**	Table 2
B3	Main desk 2	**D3**	Top of TV
B4	Top of bin	**D4**	TV stand base
B5	Foot pedal of bin	**D5**	Steering wheel toy car
B6	Trunking between doors	**D6**	Base of toy car
B7	Handle of assisted WC	**D7**	Chair
B8	Floor by exit door	**D8**	Chair
B9	Seat of rocking horse	**D9**	Floor by pillar
B10	Floor under desk	**D10**	Wall trunking
B11	Chair	**D11**	Table 3
B12	Trunking under cupboard	**D12**	Floor under table

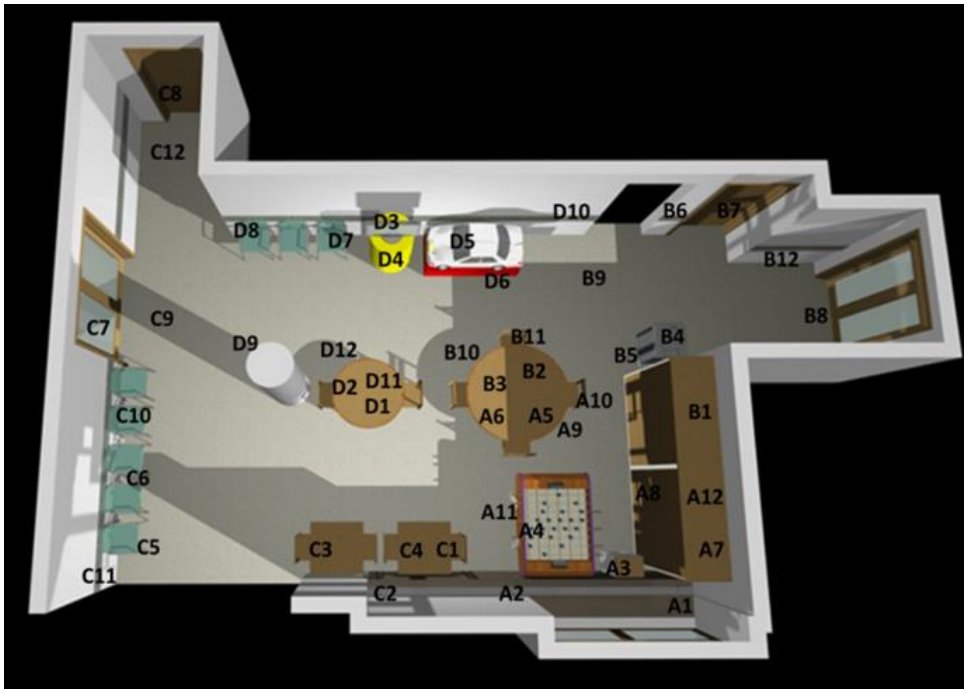


Figure 4.2: Sampling sites in the outpatient's area

Table 4.3: Sampling sites in the classroom

Sample Number	Location	Sample Number	Location
A1	Door handle	**C1**	Bookshelf
A2	Top of drawers	**C2**	Time Out' desk
A3	Bookshelf	**C3**	Windowsill
A4	Top of whiteboard	**C4**	Towel dispenser
A5	Top of speaker	**C5**	Sink
A6	Desk 1	**C6**	Tap
A7	Desk 2	**C7**	Desk 6
A8	Top of projector	**C8**	Desk 7
A9	Bookshelf	**C9**	Windowsill
A10	Floor by door	**C10**	Desk 8
A11	Light switch	**C11**	Desk 9
A12	Drawers	**C12**	Under sink
B1	Top of speaker	**D1**	Windowsill
B2	Desk 3	**D2**	Computer tower
B3	Computer keypad	**D3**	Key cupboard
B4	Pipes	**D4**	Mini projector
B5	Bookstand	**D5**	Windowsill
B6	Drawers	**D6**	Drawer
B7	Drawer front	**D7**	Desk 10
B8	Desk 4	**D8**	Desk 11
B9	Desk 5	**D9**	Desk 12
B10	Windowsill	**D10**	Coat hook
B11	Floor by drawers	**D11**	Floor by drawers
B12	Computer desk	**D12**	Pipes

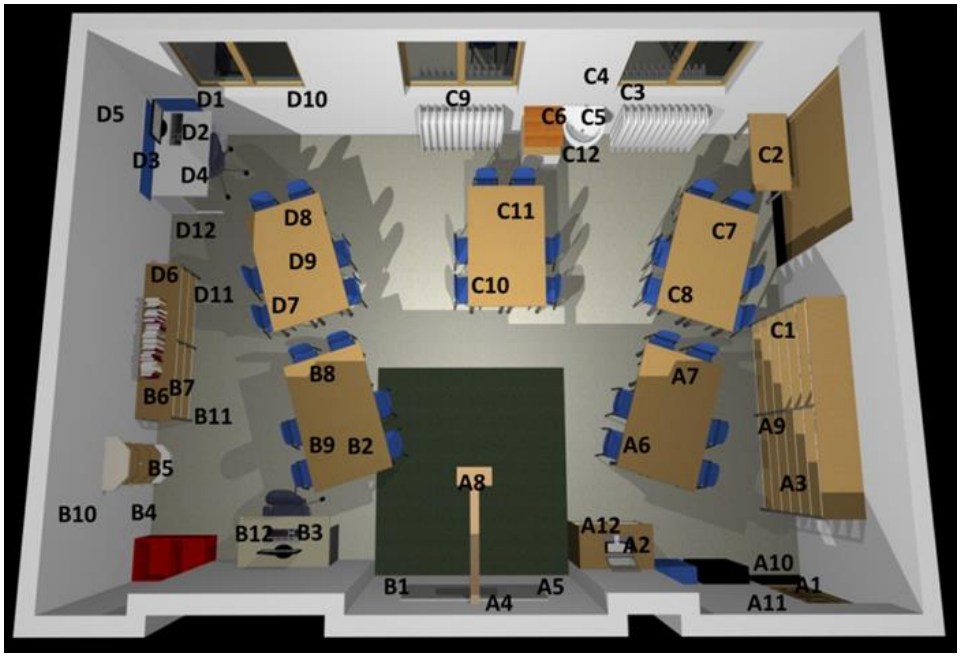


Figure 4.3: Sampling sites in the classroom

4.4.2 Culture methods

TSA contact plates and charcoal enrichment swabs were taken as outlined in sections 2.2.1 and 2.2.2.2, respectively. Colonies on TSA plates were counted and recorded as CFU per plate and pure cultures from enrichment swabs were harvested from blood agar and stored at -80 ^{O}C in glycerol for subsequent processing.

4.4.3 Identification

Gram stains were performed on isolates obtained by enrichment culture to provide basic identification (Section 2.4.1). Isolates that had a similar morphology to an *S. aureus* reference strain on agar plates and by staining were tested using the catalase test and Pastorex® Staph Plus kit as described (2.4.2.1 and 2.4.2.2).

4.4.4 DNA extraction

Glycerol stocks of pure cultures were thawed and a loop-full (approximately 10 μl) of bacterial suspension was streaked out onto blood agar. The plates were incubated at 37 ^{O}C for 24 hours. DNA extraction was carried out as in Section 2.5.1.

4.4.5 16S rRNA gene PCR

End-point PCR was carried out on the DNA extracts from all isolates from all locations using universal primers 27F and 1492R to amplify a region within the bacterial 16S rRNA gene, as described in Table 2.2.

4.4.6 Sequencing

Sequence data were analysed using the method given in Section 2.10.4.1, using the Chromas LITE V2.01 software package and on-line BLAST program.

4.5 Results

4.5.1 Total viable count results

Full sets of 48 total viable count (TVC) plates were collected from the classroom and outpatient's area. However, on Tiger ward, some sites were not available for sampling due to the presence of staff or parents. Extra samples, not in the designated sampling plan were taken, on the ward to make up for this shortfall (prefix 'E'). A total of 43 samples were taken on the ward.

Colony counts were divided into 4 categories; < 50, 50-100, 101-200 and > 200 CFU and given a colour code. TVC results obtained using contact plates for each site are presented in Table 4.4. Cells are colour-coded as described. If colonies on TSA plates had merged, TVC was estimated based on the percentage of plate that the colonies covered.

More sites in the classroom had > 200 CFU than in the other locations and more sites on the ward had < 50 CFU. However, due to the diverse nature of the locations, absolute numbers of plates taken at each location varied. The data have been corrected to account for this by expressing as percentages (Figure 4.4).

Table 4.4: Bacterial total viable counts recovered from sampling sites on a hospital ward, outpatient's area and classroom

Ward			Classroom			Outpatient Area		
Sample number	Location	CFU	Sample Number	Location	CFU	Sample Number	Location	CFU
A1	Floor under bed	146	A1	Door handle	1	A1	Windowsill	128
A2	Monitor shelf	1	A2	Top of drawers	52	A2	Heating ledge	11
A3	Chair	>200	A3	Bookshelf	>200	A3	Drawers	128
A4	Trolley top	1	A4	Top of whiteboard	>200	A4	Football table	31
A5	Monitor top	0	A5	Top of speaker	41	A5	Main table	0
A6	Drawer front	0	A6	Desk 1	57	A6	Main table 2	1
A7	Top of light	26	A7	Desk 2	3	A7	Top of cupboard 1	184
A8	Bed rail	33	A8	Top of projector	>200	A8	Front of cupboard 2	11
A9	Sink (right)	72	A9	Bookshelf	>200	A9	Under main table	146
A10	Window (right)	27	A10	Floor by door	>200	A10	Chair	29
A11	Sink (left)	26	A11	Light switch	7	A11	Floor under football table	87
A12	Bed pedal	Merged	A12	Drawers	>200	A12	Top of cupboard 2	>200
B1	Floor under bed	Merged	B1	Top of speaker	Merged	B1	Top of cupboard 3	145

Ward			Classroom			Outpatient's area		
Sample number	Location	CFU	Sample number	Location	CFU	Sample number	Location	CFU
B2	Monitor shelf	0	B2	Desk 3	28	B2	Main desk 1	2
B3	Chair	80	B3	Computer keypad	>200	B3	Main desk 2	0
B4	NO SAMPLE	-	B4	Pipes	>200	B4	Top of bin	64
B5	Monitor top	0	B5	Bookstand	>200	B5	Foot pedal of bin	37
B6	Drawer front	1	B6	Drawers	174	B6	Trunking between doors	81
B7	Top of light	8	B7	Drawer front	131	B7	Handle of assisted WC	65
B8	Bed rail	0	B8	Desk 4	18	B8	Floor by exit door	53
B9	Trolley top	67	B9	Desk 5	13	B9	Seat of rocking horse	22
B10	Window (left)	15	B10	Windowsill	>200	B10	Floor under desk	71
B11	Bed pedal	Merged	B11	Floor by drawers	>200	B11	Chair	101
B12	NO SAMPLE	-	B12	Computer desk	108	B12	Trunking under cupboard	167
C1	Floor under bed	>200	C1	Bookshelf	188	C1	Play table	1
C2	Monitor shelf	0	C2	'Time Out' desk	24	C2	Heating vent	>200
C3	NO SAMPLE	-	C3	Windowsill	49	C3	Toy	5
C4	NO SAMPLE	-	C4	Towel dispenser	>200	C4	Table 2	37
C5	Monitor top	1	C5	Sink	>200	C5	Chair leg 1	>200
C6	Drawer front	0	C6	Tap	>200	C6	Chair leg 2	159
C7	Top of light	2	C7	Desk 6	16	C7	Door	2
C8	Bed rail	52	C8	Desk 7	14	C8	Handle female WC	88

Ward			Classroom			Outpatient's area		
Sample number	Location	CFU	Sample number	Location	CFU	Sample number	Location	CFU
C9	Trolley top	0	C9	Windowsill	46	C9	Sign	17
C10	Window (left)	Merged	C10	Desk 8	7	C10	Chair	46
C11	Bed pedal	Merged	C11	Desk 9	10	C11	Corner trunking	46
C12	NO SAMPLE	-	C12	Under sink	>200	C12	Floor by female WC	98
D1	Floor under bed	>200	D1	Windowsill	89	D1	Table 1	8
D2	Monitor shelf	22	D2	Computer tower	>200	D2	Table 2	0
D3	Chair	16	D3	Key cupboard	73	D3	Top of TV	161
D4	NO SAMPLE	-	D4	Mini projector	67	D4	TV stand base	141
D5	Monitor top	8	D5	Windowsill	>200	D5	Steering wheel toy car	32
D6	NO SAMPLE	-	D6	Drawer	47	D6	Base of toy car	163
D7	NO SAMPLE	-	D7	Desk 10	1	D7	Chair	>200
D8	NO SAMPLE	-	D8	Desk 11	17	D8	Chair	23
D9	Trolley top	4	D9	Desk 12	2	D9	Floor by pillar	109
D10	Window (right)	17	D10	Coat hook	23	D10	Wall trunking	53
D11	Bed pedal	Merged	D11	Floor by drawers	>200	D11	Table 3	102
D12	NO SAMPLE	-	D12	Pipes	>200	D12	Floor under table	129
E1	Alcohol dispenser	176						
E2	TV shelf	Merged						
E3	Towel dispenser	Merged						
E4	Towel dispenser	Merged						
E5	Alcohol dispenser	139						

Green: < 50 CFU, Yellow: 50 – 100 CFU, Orange: 101 – 200 CFU, Red: > 200 CFU

Figure 4.4 shows the percentages of sites with TVCs in each category. It can be seen that the majority of TVCs obtained across all sites were low (<50 CFU). However, over half of the sites in the classroom and outpatient's and just under half in the ward were in the medium to higher categories.

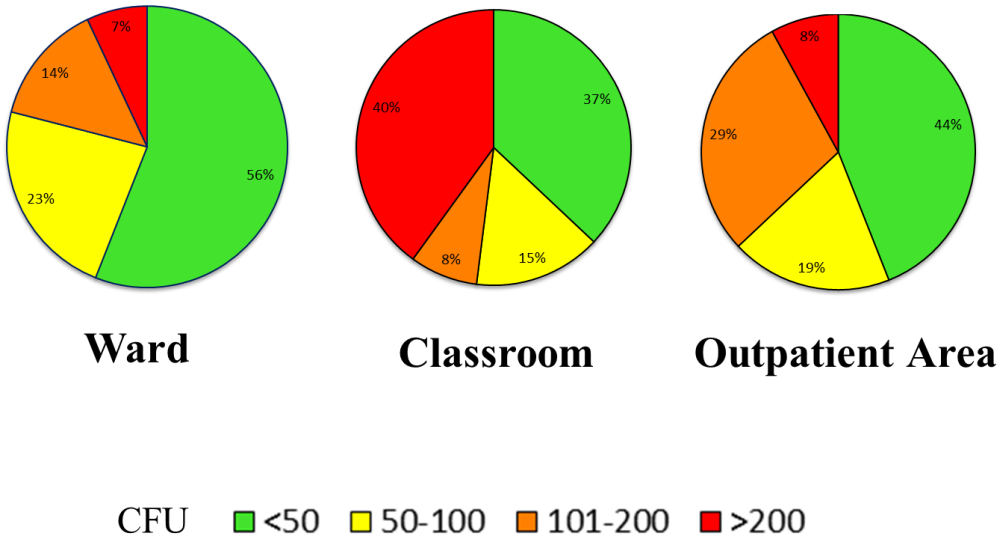


Figure 4.4: Percentage of sampling sites at each of 3 locations with bacterial total viable counts in each of 4 numerical categories.

The percentage of sites with high CFUs was much greater in the school (40 %) than the ward and outpatient's area which had similar levels of sites in this category of 7 % and 8 % respectively.

The ward had the least environmental contamination with 56 % of sites having < 50 CFU. Only 3 sites from the ward had counts > 200 CFU, two of these were floor sites and the other was a chair seat (Figure 4.5). The remainder of the sites with higher counts on the ward were items such as bed foot pedals and the tops of non-

clinical items like the shelf that held the television. All clinical items such as trolleys and monitors and items near to the beds showed low CFU recovery (Figure 4.5).

The classroom had a much higher number of sites with colony counts of > 200 CFU (Figure 4.6). The contact plates taken at many sites within the classroom, in fact, were almost confluent after 24 hours of incubation. These high TVCs tended to be found on items such as floors, bookshelves and the tops of items. Desks and student drawers in this location tended to have low colony counts of < 50 CFU.

The outpatient's area had only 4 sites that showed > 200 CFU but had a high percentage (29 %) of sites with 101-200 CFU (Figure 4.7). As observed in the classroom, desks and tables showed lower counts.

Sampling sites can be seen in Figures 4.5 – 4.7, showing CFUs recovered and the height and touch frequency of each site.

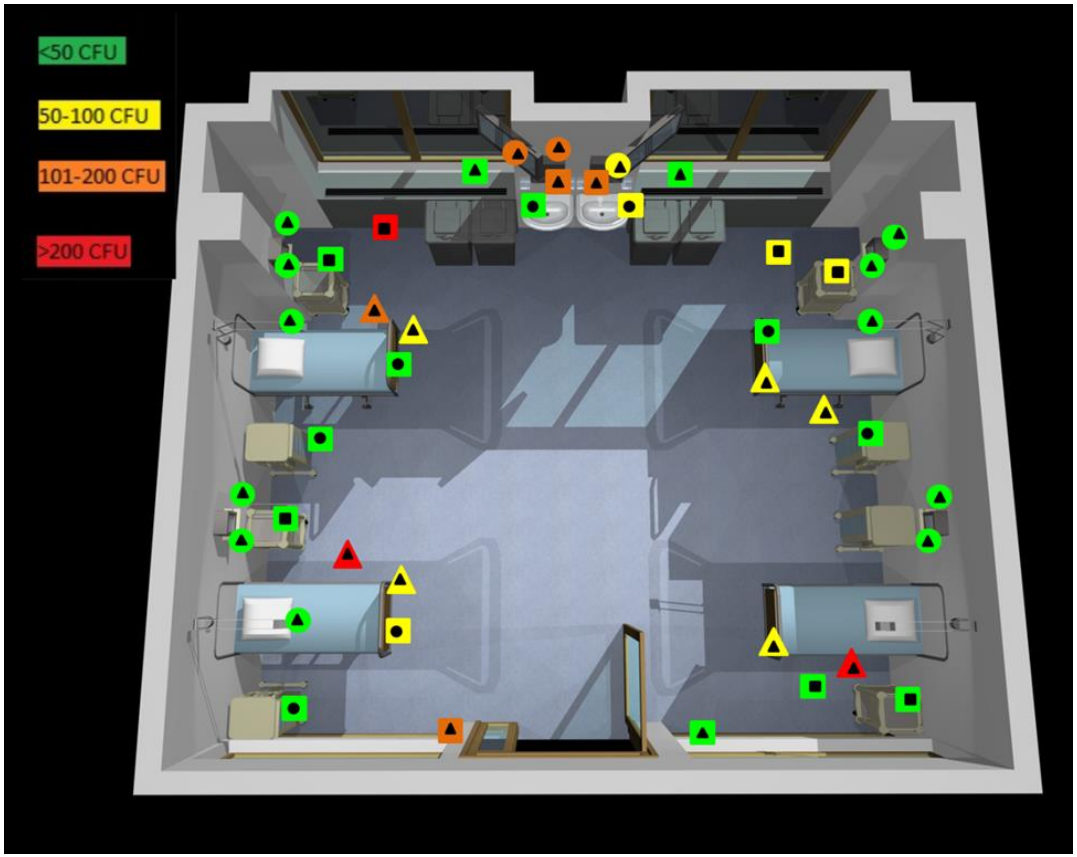


Figure 4.5: Sampling sites on the ward showing height, touch-frequency and recovered colony-forming units

Colours are indicative of CFU counts as given in the figure. The shape of the colour block corresponds to object height, circles for high (>1.5 m), squares for medium (1-1.5 m) and triangles for low (<1 m). Internal shapes correspond to touch-frequency, circles for high, squares for medium and triangles, for low.

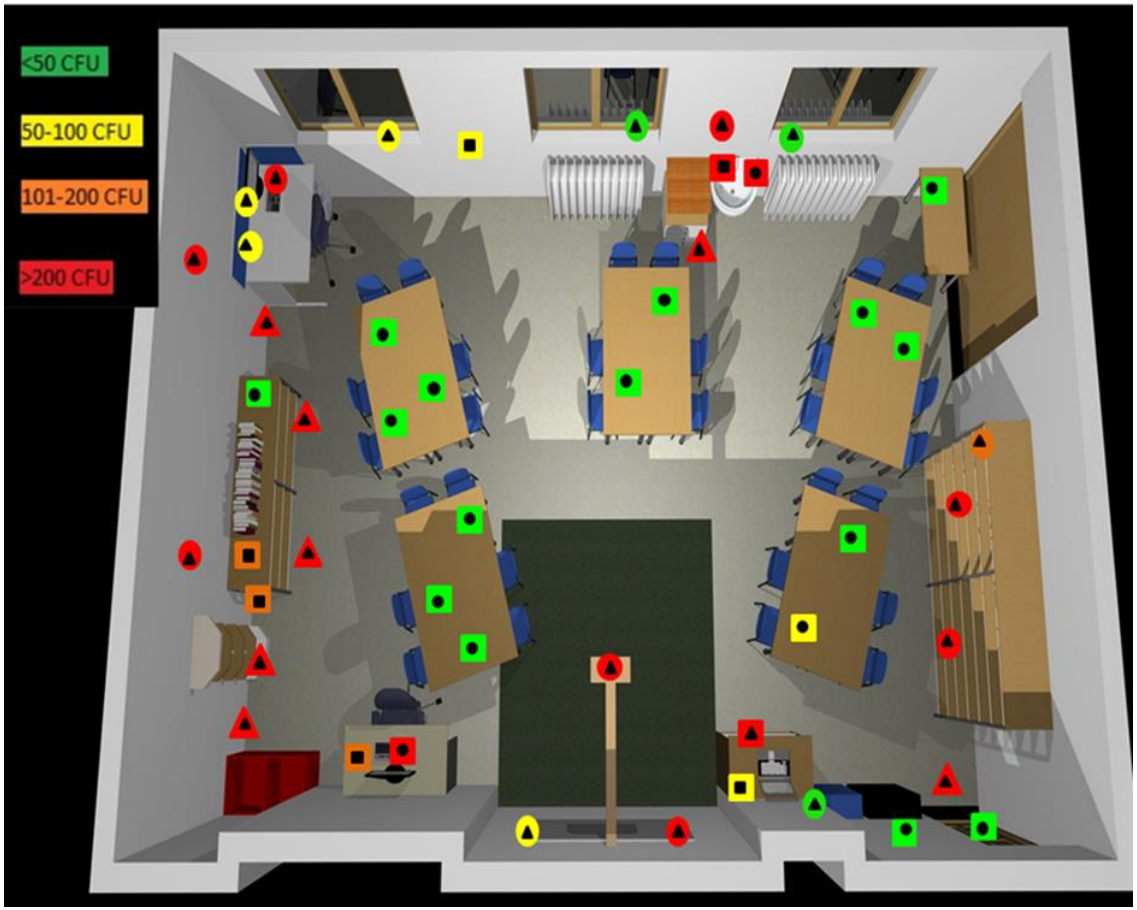


Figure 4.6: Sampling sites in the classroom showing height, touch-frequency and recovered colony-forming units

Colours are indicative of CFU counts as given in the figure. The shape of the colour block corresponds to object height, circles for high (>1.5 m), squares for medium (1-1.5 m) and triangles for low (<1 m). Internal shapes correspond to touch-frequency, circles for high, squares for medium and triangles for low.

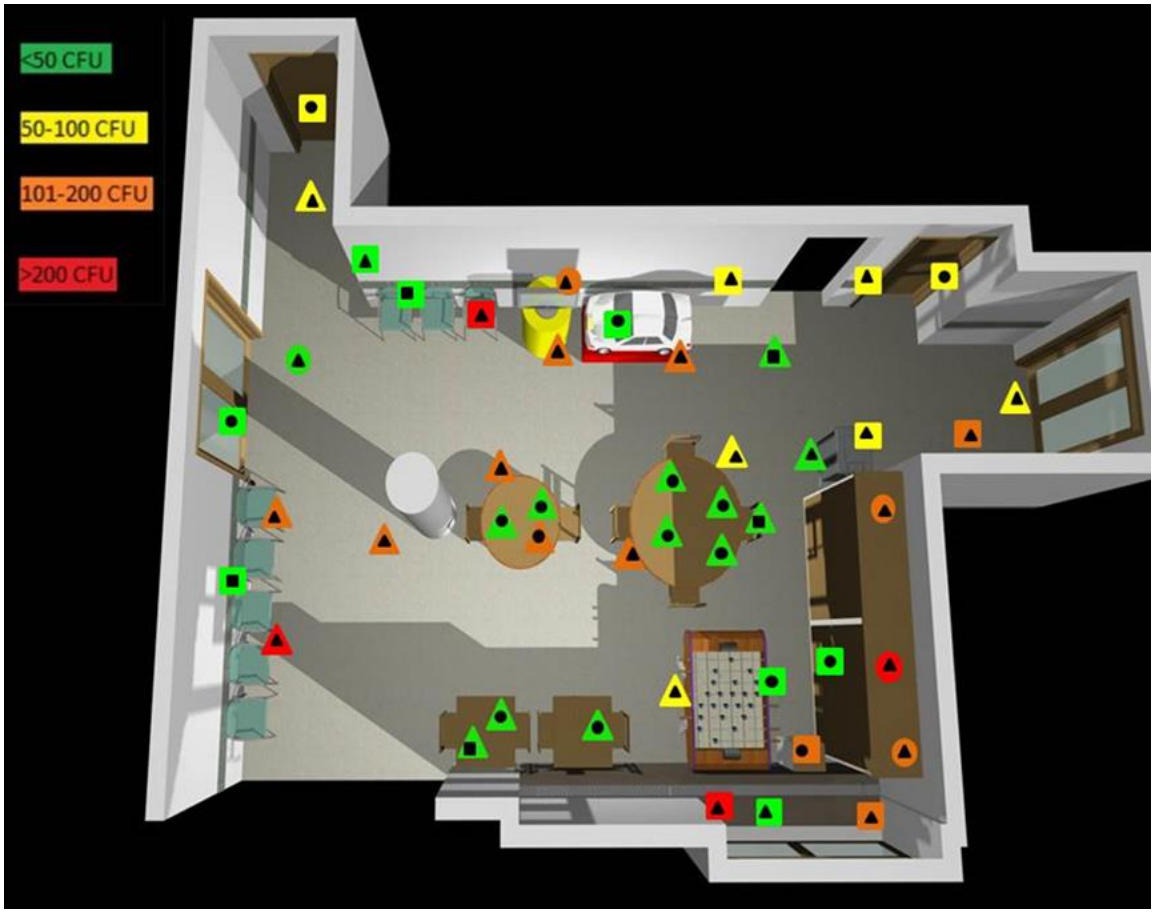


Figure 4.7: Sampling sites on the ward showing height, touch-frequency and recovered colony-forming units

Colours are indicative of CFU counts as given in the figure. The shape of the colour block corresponds to object height, circles for high (>1.5 m), squares for medium (1-1.5 m) and triangles for low (< 1 m). Internal shapes correspond to touch-frequency, circles for high, squares for medium, and triangles for low.

4.5.1.1 Total viable counts by sample site height

In order to determine if sample site height had an effect on CFU recovery, sites were categorised into low (< 1 m), medium (1-1.5 m) and high (> 1.5 m). Due to the diverse nature of the locations, equipment and items within them, absolute numbers of contact plates taken at each height varied. The data have been corrected to account for this by expressing as percentages (Figure 4.8).

Sampling sites in the high height category included shelves, window ledges and tops of cupboards. Medium level items were desks, chairs and trolley tops and low items were floors and bases of floor-standing equipment. Of the high sites sampled on the ward, 60 % had low TVC counts, of the high sites sampled in the classroom, 50 % had high TVCs and for the outpatient's area, the majority of high sites had intermediate-to-high CFUs (Figure 4.8).

Of the low sites sampled, only the outpatient's area had some sites with low TVCs, 100 % of low sites in the classroom had > 200 CFU. For the ward, low sites mostly had low-intermediate CFUs but a small percentage of sites showed higher counts. For the most part, at all locations, medium height sampling sites had low TVC recovery but the outpatient's area had a greater range of counts for this height category (Figure 4.8).

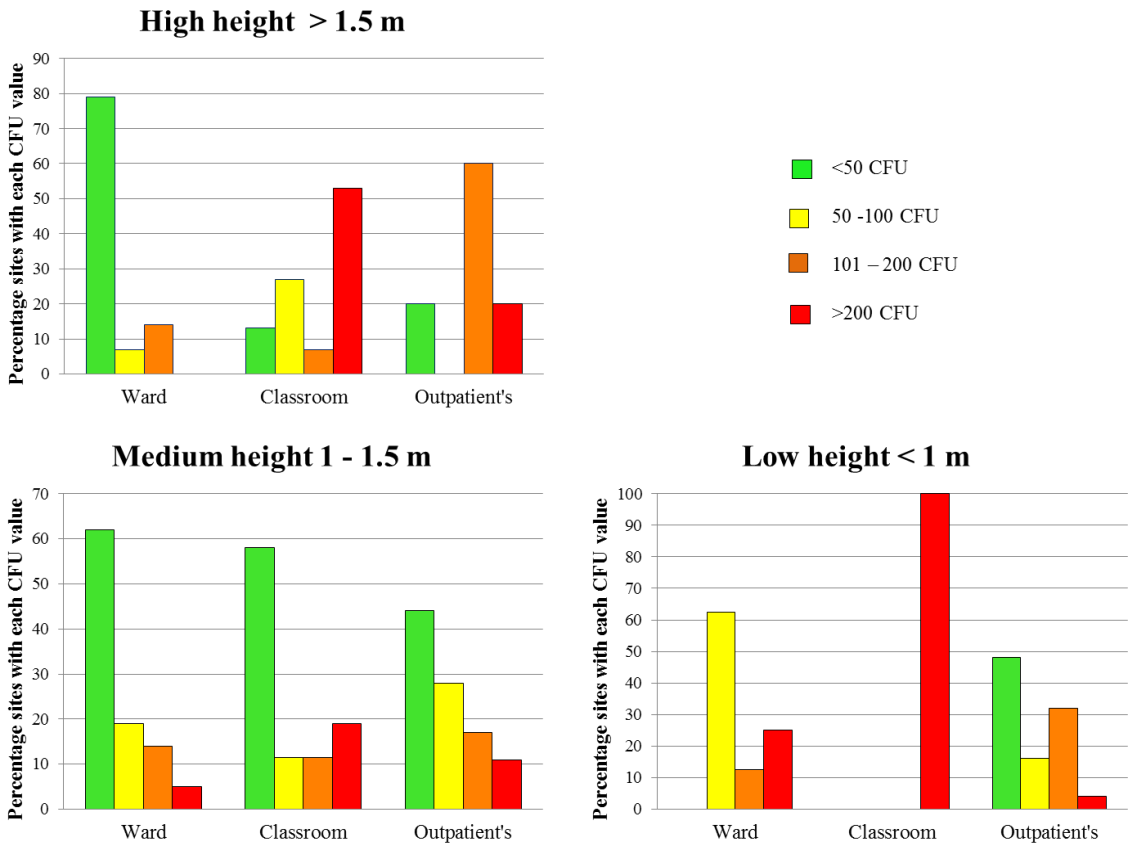


Figure 4.8: Percentage of sites sampled with bacterial colony forming units in different categories, recovered at 3 different heights on the ward, in the classroom and outpatient's area.

4.5.1.2 Total viable counts by touch-frequency

The data were arranged with regard to touch-frequency; high-touch being items such as desks or door handles and low-touch items including hard-to-reach areas such as high windowsills. Medium-touch items were items such as chairs, trolleys and drawer fronts.

High-touch sites had low CFU recovery in all locations with the majority of TVC plates falling into this category. 75 % of high-touch sites sampled on the ward and outpatient's area and 82 % of high-touch sites in the classroom had < 50 CFUs. Medium touch sites showed a range of CFUs in the ward and classroom but all medium-touch sites had < 50 CFUs in the outpatient's area. Low touch sites appeared to show no trend, showing a range of CFU recovery, except in the classroom where 67 % of low sites sampled had high CFUs (Figure 4.9).

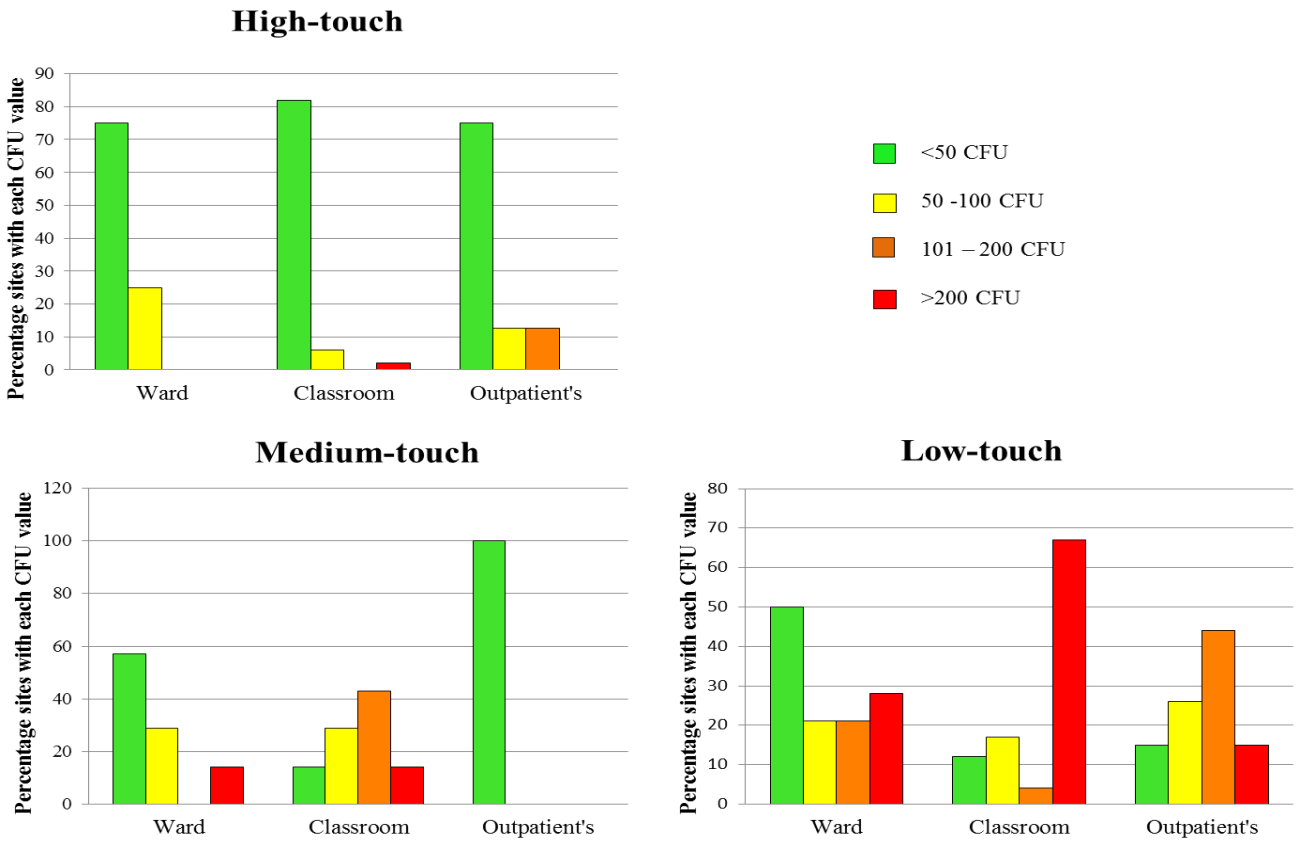


Figure 4.9: Percentage of sites sampled with bacterial colony forming units in different categories, recovered from different touch-frequency items on the ward, in the classroom and outpatient's area.

4.5.2 Sequencing identification results

Bacillus was the most frequently isolated genus in both the outpatient's area and classroom occurring at 42 % and 50 % of sites respectively (Figure 4.10). The next most frequently isolated genus in both of these locations was *Staphylococcus* occurring at 25 % and 19 % of sites respectively. On the ward, *Staphylococcus* was most frequently isolated, found at 26 % of sites and *Bacillus* was isolated from 23 %. The staphylococci were mostly coagulase-negative (CNS) in all locations but biochemical testing identified the presence of *S. aureus* on a chair on the ward, desk in the classroom and the handle of a game, toilet door handle and floor in the outpatient's area.

9 % of sites sampled on the ward had *Pseudomonas* spp. present, compared to 2 % and 4 % in the classroom and outpatient's area. Sequence data and biochemical tests confirmed the presence of *P. aeruginosa* on a sink on the ward. Enterobacteriaceae were isolated from all locations, most frequently from the outpatient's area (Table 4.5), *Klebsiella* sp. was isolated from both the classroom and outpatient's area and *Enterobacter* sp. was common to all 3 locations. *A. baumanii, E. cloacae* and *P. aeruginosa* were only identified from ward samples.

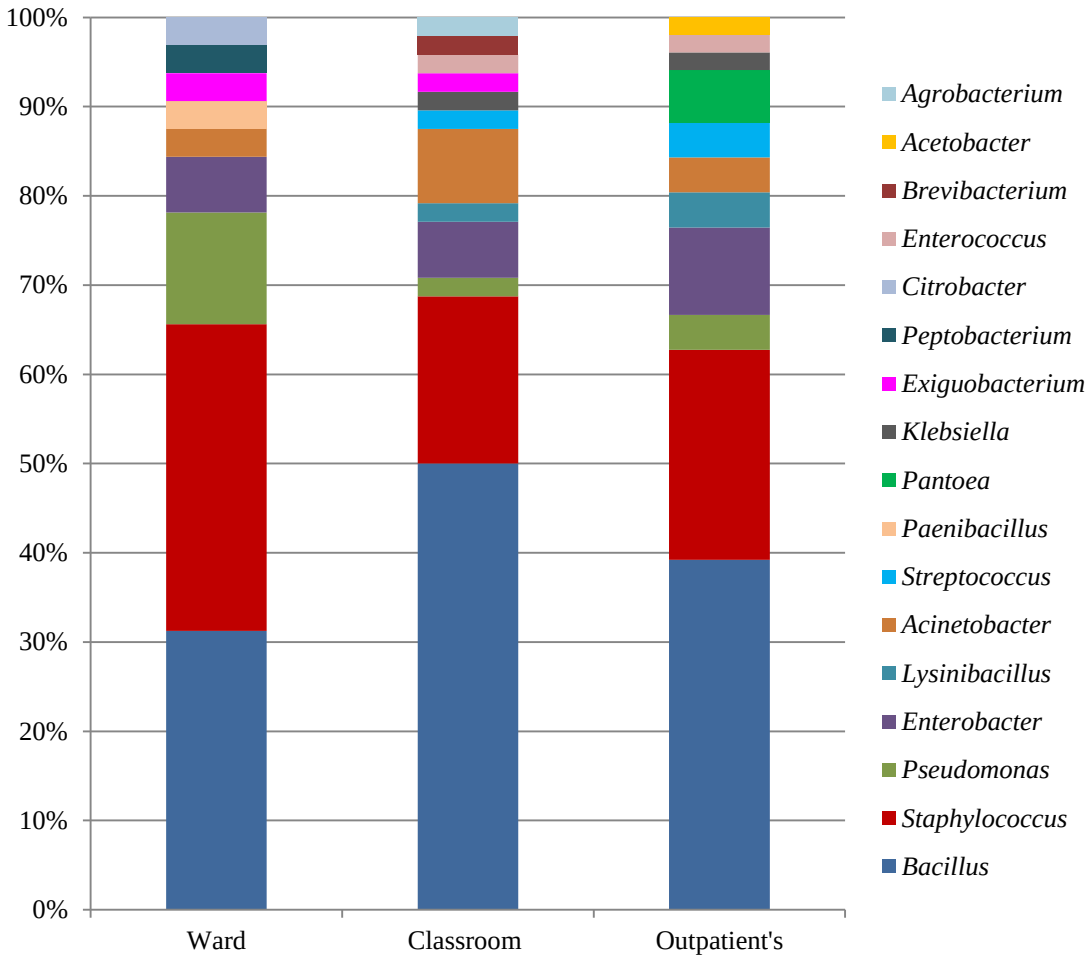


Figure 4.10: Bacterial genera and frequency of isolation from the ward, classroom and outpatient's areas.

Table 4.5: Number of sites from which selected clinically-relevant pathogens were identified in each of 3 locations: ward, classroom and outpatient's waiting area.

	Number of sites		
Identification	**Ward**	**Classroom**	**Outpatient's**
Pseudomonas aeruginosa	1	-	-
Staphylococcus aureus	1	1	3
Acinetobacter baumanii	1	-	-
Acinetobacter lwoffii	-	1	1
Enterobacter cloacae	2	-	-
***Enterococcus* sp**	-	2	1
***Streptococcus* sp**	-	1	2
***Kelbsiella* sp**	-	1	1
Other Enterobacteriaceae	3	2	8

4.5.2.1 Ward sequencing data results

A total of 9 different genera were isolated from Tiger ward at the time of sampling (Table 4.6). *Staphylococcus* was the most common genus, occurring at 26 % of sampling sites analysed and *Bacillus* occurred with similar frequency at 23 % of sites. *S. aureus* was found on a chair (A3). *Pseudomonas* DNA was isolated from 9 % of sites, with one of these isolates from a sink (A9) being identified by biochemical means as *P. aeruginosa*, other isolates could not be identified to species-level but belonged to the *P. aeruginosa* group. Other clinically-relevant bacteria identified included *A. baumanii* from a bed pedal (C11) and *E. cloacae* from the floor (D1).

Table 4.6: Identification of bacteria from the ward, showing known pathogens in red, *-confirmed biochemically.

Sample number	Sample site	Bacteria ID	Max. Identity (%)
A1	Floor under bed	*Bacillus* sp.	100
		Staphylococcus sp. (CNS)	100
A2	Monitor shelf	*Staphylococcus* sp. (CNS)	100
A3	Chair	*Bacillus* sp.	100
		*Staphylococcus aureus**	100
A4	Trolley top	*Bacillus* sp.	100
A6	Drawer front	*Paenibacillus* sp.	100
A9	Sink	*Pseudomonas aeruginosa**	100
		P. aeruginosa-group	100
A10	Window	*Staphylococcus* sp. (CNS)	100
A11	Sink	*Exiguobacterium marinum*	99
A12	Wheel of bed	*Enterobacter cloacae* complex	99
B1	Floor	*Bacillus* sp.	99
		P. aeruginosa-group	100
B5	Monitor	*P. aeruginosa*-group	100
B6	Chair	*Staphylococcus* sp. (CNS)	100
B7	Top of light	*Pectobacterium cypripedii*	98
B9	Floor	*Staphylococcus* sp. (CNS)	100
B10	Windowsill	*Staphylococcus* sp. (CNS)	100
B11	Wheel of bed	*Bacillus* sp.	100
B12	Bed pedal	*Bacillus* sp.	100
C1	Floor	*Bacillus* sp.	100
C11	Pedal	*Acinetobacter baumanii*	99
D1	Floor	*Enterobacter cloacae*	99
D3	Chair	*Staphylococcus* sp. (CNS)	99
D10	Window	*Bacillus* sp.	100
		Staphylococcus sp. (CNS)	100
D11	Bed pedal	*Bacillus* sp.	100
D12	Pedal	*Citrobacter* sp.	97
E2	TV shelf	*Staphylococcus* sp.	100
E3	Towel dispenser	*Bacillus* sp.	100
E4	Towel dispenser	*Staphylococcus* sp. (CNS)	100

4.5.2.2 Outpatient's area sequencing data results

A total of 11 different bacterial genera were isolated from the outpatient's waiting area at the time of sampling. *Bacillus* was the predominant genus, occurring at 42 % of sampling sites analysed (Table 4.7). *Staphylococcus* was the next most frequent, occurring at 25 % of sites, 3 isolates of which were identified as *S. aureus*. Clinically-relevant strains, identified to species-level, were found at 23 % of analysed sites. This number was the highest of all 3 areas sampled. Six of the clinically-relevant isolates were members of the Enterobacteriaceae family and are associated with animal or human faeces and opportunistic infection. *Klebsiella* is also a member of the Enterobacteriaceae and was isolated from the wall trunking and this genus contains a number of human pathogens. *Streptococcus, Acetobacter, Acinetobacter* and *Pseudomonas* were also identified and these genera are also known to contain members which are potential pathogens.

There was a widespread distribution of clinically-relevant bacteria identified to species-level throughout the outpatient's area. No apparent clustering occurred and they were not solely associated with sampling sites near to toilets, which may have been expected, given the number of isolates associated with the gut and faecal matter (Figure 4.11). Five of these bacteria were isolated from high-touch sites including door handles and desks.

Table 4.7: Identification of bacteria from the outpatient's area, showing known pathogens in red, *-confirmed biochemically

Sample number	Sample site	Bacteria ID	Max. Identity (%)
A2	Heating ledge	*Staphylococcus* sp. (CNS)	99
A4	Football table	*Staphylococcus aureus**	99
A6	Main table 2	*Pantoea agglomerans*	99
A7	Top of cupboard 1	*Acetobacter* sp.	99
		Staphylococcus sp. (CNS)	99
A8	Front of cupboard 2	*Staphylococcus* sp. (CNS)	99
A9	Under main table	*Bacillus* sp.	99
A10	Chair	*Staphylococcus* sp. (CNS)	99
A11	Floor under football table	*Bacillus* sp.	100
		Staphylococcus sp. (CNS)	99
		Acinetobacter sp.	96
B1	Top of cupboard 3	*Bacillus* sp.	99
B2	Main desk 1	*Acinetobacter lwoffii*	99
B4	Top of bin	*Bacillus* sp.	99
		Staphylococcus sp. (CNS)	99
B5	Foot pedal of bin	*Bacillus* sp.	99
B6	Trunking between doors	*Bacillus* sp.	99
		Pseudomonas sp.	99
B8	Floor by exit door	*Bacillus* sp.	99
		Lysinibacillus sp.	99
B9	Seat of rocking horse	*Staphylococcus* sp. (CNS)	100
		Pseudomonas luteola	99
B10	Floor under desk	*Staphylococcus* sp. (CNS)	99
		Streptococcus sp. (bovis group)	100
B11	Chair	*Bacillus* sp.	99
		Lysinibacillus sp.	99
B12	Trunking under cupboard	*Bacillus* sp.	99
		Staphylococcus sp. (CNS)	99

Sample number	Sample site	Bacteria ID	Max. Identity (%)
C1	Play table	*Bacillus* sp.	94
C2	Heating vent	*Bacillus* sp.	99
		Pseudomonas sp.	99
		Enterococcus faecalis	99
C3	Toy	*Pantoea* sp.	99
C5	Chair leg 1	*Bacillus* sp.	100
C6	Chair leg 2	*Bacillus* sp.	99
		Staphylococcus sp. (CNS)	99
C8	Handle female WC	*Bacillus* sp.	99
		Enterobacter ludwigii	99
		*Staphylococcus aureus**	99
C10	Chair	*Staphylococcus* sp. (CNS)	99
C11	Corner trunking	*Staphylococcus* sp. (CNS)	99
C12	Floor by female WC	*Enterobacter hormaechei*	99
		*Staphylococcus aureus**	99
D1	Table 1	*Enterobacter* sp.	99
D3	Top of TV	*Lysinibacillus* sp.	99
D4	TV stand base	*Bacillus* sp.	99
		Pantoea dispersa	99
D6	Base of toy car	*Bacillus* sp.	99
D7	Chair	*Bacillus* sp.	99
		Enterobacter asburiae	98
		Staphylococcus sp. (CNS)	99
D8	Chair	*Streptococcus* sp. (bovis group)	99
		Bacillus sp.	99
D9	Floor by pillar	*Bacillus* sp.	99
D10	Wall trunking	*Klebsiella* sp.	97
		Bacillus sp.	99
D12	Floor under table	*Enterobacter ludwigii*	99
		Bacillus sp.	99

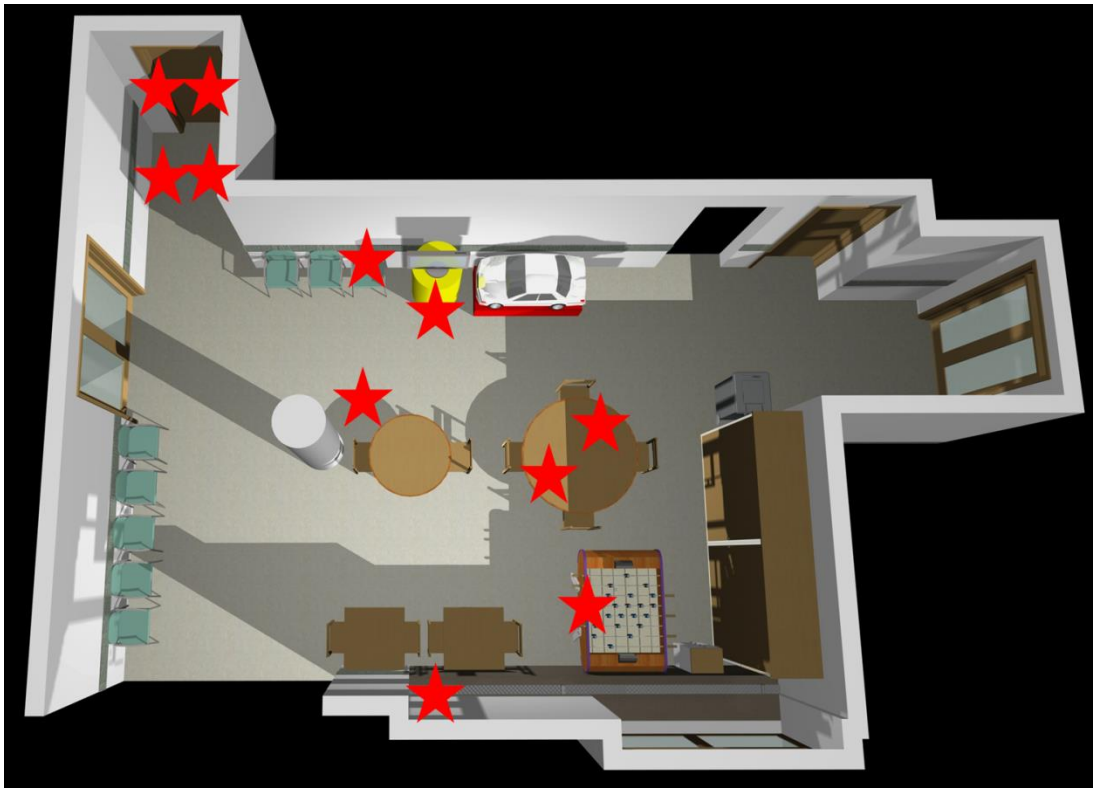


Figure 4.11: Distribution of clinically-relevant bacterial species in the outpatient's waiting area at GOSH

4.5.2.3 Classroom sequencing data results

Bacillus was the most frequently isolated genus from the classroom samples, occurring at 50 % of sampling sites analysed (Table 4.8). *Staphylococcus* was again the next most frequent, occurring at 19 % of sites. *S. aureus* was present on a desk, as confirmed by biochemical methods. *Klebsiella* sp. and *Enterococcus faecalis / faeceium* were found on tops of speakers and *Enterobacter* sp. was found on a light switch and nearby drawers.

Table 4.8: Identification of bacteria from the classroom, showing known pathogens in red, *-confirmed biochemically

Sample number	Sample site	Bacteria ID	Max. Identity (%)
A2	Top of drawers	*Exigubacterium*	99
A3	Bookshelf	*Bacillus* spp.	99
		Streptococcus sp.	99
A4	Top of whiteboard	*Bacillus* sp.	99
		Enterococcus sp.	98
A5	Top of speaker	*Bacillus* sp.	98
		Klebsiella sp.	97
A6	Desk 1	*Staphylococcus aureus**	98
A9	Bookshelf	*Bacillus* sp.	95
A10	Floor by door	*Bacillus* sp.	98
A11	Light switch	*Enterobacter* sp.	97
A12	Drawers	*Enterobacter* sp.	97
B1	Top of speaker	*Enterococcus faecalis / faeceium*	100
B2	Desk 3	*Bacillus* sp.	92
B3	Computer keyboard	*Bacillus* sp.	100
B4	Pipes	*Bacillus* sp.	100
		Lysinibacillus fusiformis	100
		Staphylococcus sp. (CNS)	100
B5	Bookstand	*Bacillus* sp.	100
B6	Drawers	*Bacillus* sp.	99
B7	Drawer front	*Bacillus* sp.	99
B9	Desk 5	*Acinetobacter* sp.	97
B10	Windowsill	*Bacillus* sp.	100
B12	Computer desk	*Bacillus* sp.	100
		Staphylococcus sp. (CNS)	100
		Acinetobacter lwoffii	99
C4	Towel dispenser	*Bacillus* sp.	100
C5	Sink	*Acinetobacter johnsonii*	99

Sample number	Sample site	Bacteria ID	Max. Identity (%)
C7	Desk 6	*Staphylococcus* sp.	99
C9	Windowsill	*Acinetobacter johnsonii*	99
		Bacillus sp.	99
C10	Desk 8	*Agrobacterium* sp.	99
C11	Desk 9	*Pseudomonas* sp.	99
		Staphylococcus sp. (CNS)	99
C12	Under sink	*Bacillus* sp.	99
D1	Windowsill	*Staphylococcus* sp. (CNS)	99
		Brevibacterium frigoritolerans	100
D2	Computer tower	*Bacillus* sp.	99
D3	Key cupboard	*Bacillus* sp.	99
		Staphylococcus sp. (CNS)	99
D4	Mini projector	*Staphylococcus* sp. (CNS)	99
		Bacillus sp.	100
D5	Windowsill	*Bacillus* sp.	99
D6	Drawer	*Bacillus* sp.	99
D8	Desk 11	*Staphylococcus* sp.	99
		Bacillus sp.	99
D10	Coat hook	*Bacillus* sp.	99
D11	Floor by drawers	*Bacillus* sp.	99

4.6 Discussion

The main source of microbial particles in indoor environments such as homes, hospitals, and schools is the presence of humans (10), (48), (49). The outpatient's area has a high frequency of transient human use with approximately 350 patients passing through per day, each with parents, outside clothing, bags, shoes and various food items. There are toilets and a baby-changing area in this location and

consequently, a large number of individuals enter the space from other areas of the hospital to use these facilities. The classroom is occupied by approximately 30 children for much of the day and despite this being less than the outpatient's area, contrasts with the lower volume of people on the ward and may explain the differences in overall TVC counts. Within the section of the ward analysed, there was space for only four patients and their parents, and there were regular visits from a small team of doctors and nurses. It has previously been shown that greater human traffic appears to be associated with higher rates of microbial contamination (44) and the data presented in this study appear to support this.

The classroom had higher bacterial counts overall than the other 2 locations, meaning that occupancy alone could not account for levels of contamination. The lower numbers of bacteria present in the hospital environments and lower counts on the ward than in the outpatient's area could be due to other factors. For example, the school was not subject to the same rigorous cleaning regimes as the hospital environments. Cleaners vacuumed the classroom carpet and cleaned easy-to-reach designated areas once a day. Areas with less easy access such as bookcases and high windowsills were not routinely cleaned. All sites that were cleaned less frequently such as bookshelves, pipes, high windowsills, tops of projectors and book stands had > 200 CFUs. This was in contrast to the desks, all of which had < 50 CFU except one, which had 57 CFUs. Other objects that were cleaned less frequently than daily such as the floor, computer key pads and the tops of items had intermediate to high CFU counts. This suggests that without regular cleaning, bacteria can accumulate on

objects. The presence of carpets and other soft furnishings is known to contribute to higher bacteria load within indoor environments (55), (62) and this, along with less frequent cleaning may account for higher numbers of bacteria being present in the classroom.

A similar pattern was observed in the outpatient's area, with objects such as the windowsill, tops of cupboards, chair legs and wall-mounted plastic trunking having higher numbers of bacteria than other items. These sites may well be over-looked and not as frequently cleaned as play areas or easier to reach sites. The pattern was once again repeated on the ward but with lower overall CFU counts observed. High-touch items or those within easy reach had lower CFUs than other sites. Gaudart et al. reported that items > 1.2 m high were often more heavily contaminated in an ICU than lower items (45), also suggesting that difficult to reach areas may not be cleaned as well or as frequently as other sites. The only sites sampled that did not fit this pattern in the current study were floors. Despite being frequently cleaned, particularly in the hospital, floors showed high bacterial counts and this was also observed by Gaudart et al. (45).

On the ward, infection control practices such as hand washing, cleaning and wearing of appropriate clothing are strictly observed. As such, the people present are aware of reducing microbial contamination and this may contribute to the lower numbers of bacteria found in this area.

4.6.1 Sample height and touch-frequency

It might be expected that higher bacterial counts would be found in higher, harder to clean areas and this appears to be the case in the school and outpatient's area. However, low areas such as floors, which may be frequently cleaned, also had high bacterial counts. The continual replenishment of bacteria by footfall, trolleys and the settlement of bacteria from skin or clothing may account for high CFUs observed on floors. It was observed that when beds were present on the ward, floor areas under the beds were not mopped during routine cleaning.

High-touch objects may be expected to be more highly contaminated but in all locations, the majority of high-touch items had low bacterial counts. On the ward and the outpatient's areas, these high-touch items tended to be the focus for cleaning. On the ward, for example, clinical staff are required to wipe down items such as trolleys, tables and bedrails throughout the day. This likely results in reduced overall contamination. In the outpatient's area, play co-ordinators are often present and they are also responsible for wiping high-touch areas. It would appear from the data that high-touch areas in the classroom such as desks may also be the focus for cleaning.

4.6.2 Sample site material

The data could not be assessed with regards to the material type of each object sampled due to the high variability of materials between and within locations. However, the presence of many more fabrics in the classroom, rugs and curtains for

example, may lead to TVCs being higher than in other locations. Gravesen et al. found that overall concentrations of airborne bacteria in schools and offices doubled when carpets were in place than when not (242). This was not observed in another study by Bartlett et al., although they did report higher numbers of airborne bacteria when more clutter was present (36). Where the ward and outpatients floor may be comprised of the same material, this is likely to be the only site that is directly comparable between all locations. Whilst it is acknowledged that materials retain or release bacteria differently, it was beyond the scope of this preliminary study to address this.

4.6.3 Bacterial identification

Staphylococcus and *Bacillus* were the most frequently isolated genera in all locations. This may have been expected as they are associated with the skin and environment respectively and are ubiquitous. The observations are in agreement with other culture-based studies regarding indoor bacteria. Okten and Asan found the predominant bacteria in a paediatric hospital to be Gram positive cocci (63 %) followed by Gram positive-bacilli (34 %) (42). Obbard and Fang reported similar results, finding skin commensals such as CNS and *Acinetobacter*, and environmental taxa including *Pseudomonas* in a hospital (35). Gut-associated microorganisms such as *E. cloacae* were observed in all locations, more so in the classroom and outpatient's area, suggesting the presence of faecal material in these locations. Such organisms may not be pathogenic for immunocompetent children in a school but in healthcare environments, they may be clinically-relevant. Importantly, people

passing through the outpatient's area, for example, may travel to other areas of the hospital, leading to potential spread of pathogens to susceptible people.

Very little research has been conducted with regard to surface sampling and microbial community analysis in the school environment; therefore it is difficult to compare results obtained in this study with the literature. Most investigative studies have focussed on indoor air quality and the presence of bacteria or microbial components in the air (31), (47), (151). However, surface bacteria may reflect airborne communities and Bartlett et al. reported *Micrococcus*, *Staphylococcus* and *Bacillus* to be the predominant genera in classroom air (36). They found no Gram negative species however, possibly due to damage caused by sampling. Qian et al. also reported skin-associated taxa being present in air (47). The similarity of surface and air samples may suggest a link between them. Further studies must be conducted, simultaneously sampling both air and surfaces however and this is reported in Chapters 5 and 6.

Bacterial taxa may have been underestimated in this study, given that enrichment culture was used prior to sequencing. This study was intended to be a preliminary assessment of bacteria within 3 environments in order to determine information regarding any similarities or differences but also to inform a more long-term sampling strategy. However, despite the limitations of culture methods, the results presented do show important differences in the 3 locations investigated. They also show that a variety of bacterial taxa can be isolated, even with a method known to

underestimate diversity. The aims of the chapter were achieved, in that the study provided results to inform the remainder of the work.

4.7 Conclusion

The concept of a characteristic microbial community associated with different indoor environments is worth further thought. It might be that the microbial community structure in each of these environments is driven by a number of parameters similar to classic microbial environments in the gut or oral cavity, for example. Population shifts due to changing parameters such as nutrient availability or desiccation, are well documented for many microbial systems and may occur in larger areas such as indoor environments.

This study highlights the difficulty in preventing the accumulation of potentially pathogenic microorganisms in environments frequented by children. There is a current emphasis by the World Health Organisation on utilising the 'Five moments for hand hygiene' strategy which takes into consideration cleaning hands when touching patient surroundings in healthcare environments (243). This strategy might help to limit spread of infectious organisms to susceptible individuals in clinical environments; however it will remain unlikely that surfaces will ever be totally free from microbial presence. The concepts behind environmental cleaning and hand hygiene strategies are useful and could be of benefit if disseminated out of the healthcare environment into the community. This could extend to schools, where high levels of bacteria have been reported. Perhaps a more global microbial

ecological view is required if we are to modulate the potentially dangerous microbial environments frequented by at risk individuals such as children.

Overall, the hospital environment appears to have less environmental bacterial contamination than a classroom and this may be due to cleaning and types of objects present. However, different areas within the same hospital have differences in bacterial numbers, taxa and distribution. Height seems to have a small effect on CFU recovery in each location, as does touch frequency.

The techniques used in this small comparative study appeared sufficient to capture a broad range of organisms from the environment and were considered suitable for the subsequent studies along with the addition of techniques such as processing swabs by molecular methods and air sampling. Different bacterial taxa were isolated from all environments and demonstrated the presence of known pathogens; therefore it was assumed that with more sensitive techniques, more diversity would be revealed.

5. Investigating the presence of viruses in the environment

5.1 Introduction

Outbreaks of infection of viral aetiology are common in healthcare environments. This is particularly true of paediatric hospitals as children are more likely to be admitted with infections that are related to community epidemics, such as those caused by respiratory and gastrointestinal viruses. Viral disease may not be the primary reason for admission but the patient may carry virus with them, remaining asymptomatic and shedding infectious particles for lengthened periods of time (167), (208), (209), (210), (244). This is also true for visitors and healthcare workers, who can unwittingly bring viruses into the environment.

Nosocomial outbreaks of viral disease have significant financial impacts on the NHS. For example, Norovirus (NV) outbreaks are estimated to cost over £100 million in high incidence years (245), (246). In addition to being a financial burden, virus outbreaks can affect staffing levels on wards and have substantial impacts on clinical outcome, recovery time and psychological health of patients. Acquisition of rotavirus (RV), for example, is associated with increased duration of hospital stay,

increasing costs, decreased available beds and increasing pressure on staff. Hospitalization of children can have long-term psychological effects, including post-traumatic stress disorder (PTSD), intrusive thoughts and avoidance behaviours following discharge (247).

Great Ormond Street hospital is a large paediatric hospital and centre for heart and brain surgery in addition to being a specialist oncology treatment centre. As such, a large proportion of patients are present with congenital conditions, immunosuppression due to various factors; oncological conditions, primary immune deficiencies, transplant recipients and those on immunosuppressive drugs for rheumatologic conditions and severe asthma and co-morbidities that make them particularly vulnerable to viral disease. For example, respiratory syncytial virus (RSV) infection has long been known to present more severely in children with congenital heart disease, increasing the likelihood of assisted ventilation being necessary and increasing mortality rates (248). Also, NV has been shown to be the most common pathogen associated with gastroenteritis in paediatric patients with immune deficiencies (244).

Given the high infection rates and high transmissibility of viruses and the large number of susceptible hosts in the location under investigation, it was appropriate to conduct an assessment of viral contamination of the environment.

5.2 Clinically significant viruses in paediatrics

5.2.1 Norovirus

Norovirus is a single-stranded RNA (ssRNA) virus that does not have a viral envelope. Five genotypes are currently recognised with genotypes GI, GII and GIV containing human pathogens (249). Clinical symptoms of NV infection include gastroenteritis and low-grade fever with recovery of symptoms in 12-72 hours. Prolonged shedding of viral particles occurs after infection and can lead to substantial environmental contamination. Young children, older adults and the immunocompromised have higher morbidity and mortality rates than other persons who become infected. NV has high infectivity and a low dose of < 10 virions is required for infection (250).

NV particles have been isolated from the air (20) and it is thought the virus is transmitted as airborne droplets from vomiting episodes that enter the oral mucosa, as well as via the faecal-oral route and contact with contaminated surfaces (11). Outbreaks tend to occur in the winter and are common in healthcare facilities, frequently resulting in the closure of wards. NV is stable in the environment and relatively resistant to commonly used disinfectants (11), (251). Due to the lack of a widely used cell culture system for human NV, detection relies on nucleic acid amplification techniques (250).

5.2.2 Adenovirus

Adenovirus (AV) is a non-enveloped dsDNA virus. Fifty-seven serotypes have currently been identified and categorised into subgroups A-G. Subtypes A and F cause gastrointestinal infection, B, C and E cause respiratory tract infection and subgroup D causes epidemic keratoconjunctivitis (252), (253).

Infections with AV tend to occur in the first 5 years of life and generally cause self-limiting and mild symptoms in the immunocompetent host, including pharyngitis, gastroenteritis and otitis media (253), (254), (255). However, in the immunocompromised host, more severe symptoms often develop and can lead to poor outcomes, particularly in stem-cell and solid organ transplant recipients (253), (254), (256). Rates of infection in stem cell transplant recipients range from 5 to 37 %, with the highest number in paediatric patients (255). Once excreted into the environment, AV can remain infectious for up to 35 days (257).

Nosocomial AV outbreaks have been reported in hospital settings with equipment being associated with transmission, particularly in the case of epidemic keratoconjuncitivitis (258). Direct transmission via droplets is the most common method of transmission but the faecal-oral route has also been reported (253), (259). Virus particles can be shed in the stool or nasopharyngeal secretions for weeks to months after infection (253), (257).

5.2.3 Respiratory Syncytial Virus

RSV is an enveloped, ssRNA virus belonging to the family Paramyxoviridae (260). Currently, two major subgroups are recognised; RSV-A and RSV-B, with RSV-A generally being the most prevalent. It is a major cause of lower respiratory tract infections (RTI) in children, causing bronchiolitis and pneumonia and peaking in the winter months (260), (261), (262). Figure 5.1 shows the latest Health Protection Agency (HPA) data on UK cases of RSV and it can be seen that the majority of reported cases occur in young children, especially those less than 4 years of age. RSV infection is the most common cause of hospitalisation of children with respiratory illness and between 53,000 and 199,000 deaths are reported globally per year (261). Symptoms are clinically very similar to influenza A infection therefore it is likely that RSV infection is under-reported (263). RSV is reported not to be transmitted via the aerosol route as might be expected of a respiratory virus, but by contact transmission (264).

Data regarding RSV survival time on surfaces is lacking but various studies have been conducted investigating the relationship between RSV infections and meteorological conditions (261), (262), (265), (266).

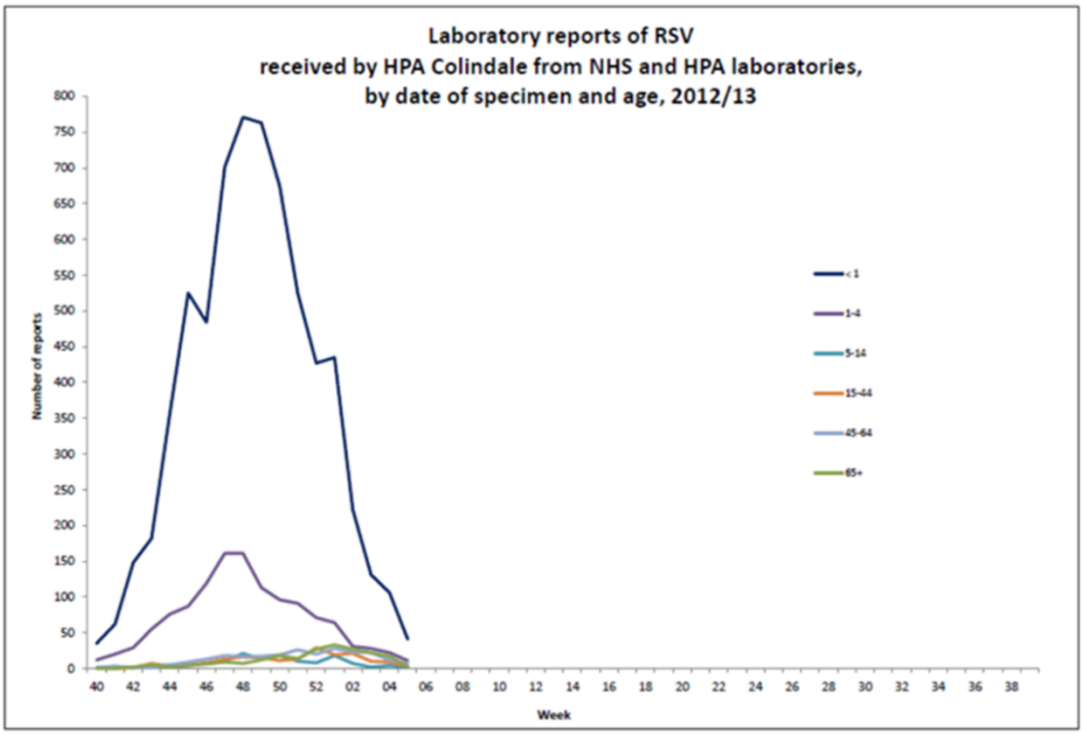


Figure 5.1: Health Protection Agency reports of Respiratory syncytial virus cases 2012/13 (267)**.**

Temperature and atmospheric pressure appear to be the most important factors associated with the activity of RSV and the number of reported infections (268). Rechsteiner et al. experimentally aerosolised RSV and found that after 1 minute at 20 ^{O}C, the droplets were most stable at 40 % RH (265). They observed that the virus survived best at lower RH than other viruses such as measles, for example (265). This could have been due to a number of factors however and experimental aerosolisation and sampling experiments such as this do not accurately reflect field conditions. This is demonstrated in a study by Nair et al. in their investigation into whether meteorological factors can predict RSV outbreaks. They recorded peak activity of RSV at around 63 % in outdoor air. They also acknowledged that the transmission of RSV and other respiratory viruses is likely multifactorial and cannot

be correlated to just one physical factor (261). Yusuf et al. did find however that RSV activity was greatest at 45 – 65 % RH in a field-based study across 9 cities (266). The experimental data obtained by Rechsteiner et al., Yusuf et al. and possibly others, may have implications for indoor transmission. If RSV truly does survive better at temperatures of around 20 ^{O}C and RH of 40 %, these conditions are frequently reached in hospital and other indoor environments and coupled with potentially infected people in close proximity to each other, could facilitate transmission of RSV.

5.2.4 Human Metapneumovirus

Human metapneumovirus (hMPV) is an enveloped, ssRNA virus, belonging to the family Paramyxoviridae. It was first isolated from children with RTI in 2001 (269) and is thought to be the second most common cause of childhood RTI, after RSV (270). Despite only being recently isolated, research has shown that the virus had been circulating for at least 50 years prior to its discovery and almost 100 % of children under 5 years of age have been exposed to it (271), (272). Clinical symptoms range from coughs to flu-like symptoms and severe respiratory distress and seizures (270), (271), (273) and can affect both the upper and lower respiratory tracts, mostly in the elderly, young and immunocompromised (274). The virus can lead to lower respiratory tract complications in stem-cell transplant recipients, 5 % of whom become infected with hMPV post-transplant (274). The mechanisms of transmission are yet to be discovered for this virus but it is suggested that contact transmission is the primary mechanism either by hands or via fomites (273).

5.2.5 Rotavirus

Rotavirus (RV) is a non-enveloped, double-stranded RNA virus of the family Reoviridae and is up to 100 nm in size (275), (276). Seven groups of RV are currently recognised (Groups A – G) but only groups A, B and C are known to cause human disease, with group A being the most common (277). The majority of cases of childhood severe acute diarrhoea are caused by RV and it is the most common reason for hospitalisation due to diarrhoea in younger children (276), (277), (278). A range of symptoms can occur during RV infection; the patient may be asymptomatic or may suffer from severe diarrhoea, leading to dehydration and possibly death (276). It is estimated that approximately 570,000 children die each year from RV-associated dehydration (277), (276). Approximately 100 – 1000 virions can be shed per millilitre of stool during diarrhoea episodes (277). Given that the infectious dose of RV is between 10 and 100 virions, it is unsurprising that it spreads rapidly and the environment becomes heavily contaminated during outbreaks (277), (279). Respiratory symptoms occur in 30 – 50 % of infected children and RV RNA has been isolated from the air, with aerosolisation being recognised as a means of disease transmission (279). Transmission primarily occurs via the faecal-oral route but the virus survives for prolonged periods on surfaces and fomites are also a recognised transmission route, (278), (280), (281).

5.2.6 Influenza

Influenza viruses are enveloped ssRNA viruses from the family Orthomyxoviridae and are approximately 100 nm in diameter (282). Three types of influenza virus are known; A, B and C. Influenza A has 15 sub-types and causes annual epidemics, whereas influenza B circulates less frequently (282). During community outbreaks, influenza has the highest attack-rates (up to 70 %) in children from the ages of 5 – 18 years of age (282). Hospital-acquired influenza has been reported in a number of ward types (283) and results in increased length of stay, mortality and as with other HCAIs, increased financial loss (284). During the H1N1 influenza pandemic in 2009, rates of hospital-acquired influenza infection increased and this lead to cases of severe illness and a number of deaths (283).

Influenza transmission occurs via respiratory droplets that are ejected into the air by coughing and sneezing. These droplets are either directly inhaled by or absorbed by mucous membranes of a new host or can deposit on surfaces and be indirectly transmitted via the hands (285), (286). The transmission and survival of influenza virus in the environment has been extensively studied, more so than any other human pathogenic virus (285), (286), (287). Despite this, nosocomial and community outbreaks are still common and reports show conflicting information about temperature, humidity levels, UV-light exposure and other factors on the survival and transmissibility of the virus (287), (288). This shows that the acquisition of viral disease is incredibly complex and must rely on host factors as well as environmental conditions.

5.2.7 Other viruses

The viruses discussed above are only a very small selection of viruses that are of significance in paediatric healthcare environments. Among others, measles, Varicella zoster virus (VZV), parainfluenza viruses, bocavirus, sapovirus and astrovirus are all of concern and have the potential to cause nosocomial outbreaks. Cytomegalovirus (CMV) is the most commonly acquired congenital infection and the majority of cases are subclinical but infection or activation of latent disease during pregnancy can cause severe birth defects such as deafness (289), (290) and as such, control of transmission is important. CMV has been isolated from saliva and can cause pneumonia (289), virus can be shed for months to years after primary infection. Infected neonates can shed virus for up to 6 years (290). CMV rarely causes disease in the immunocompetent but cases of severe CMV pneumonia in otherwise healthy children have been reported (291), (292).

The presence of immunocompromised children and children who have pre-existing conditions make the presence of any virus, not just those that cause large-scale outbreaks, a concern for health and clinical outcome.

5.3 Viruses in the environment

As the concept of different environmental and human microbiomes is gaining popularity and more work is being carried out to identify bacterial and fungal components, references to the ‘virome’ are also beginning to appear. The use of

powerful NGS technologies is allowing the identification of previously unknown viruses and there is, understandably, great interest in the human 'virobiota' (293), (294). Environmental virome NGS investigation is currently largely focussed on samples such as sewage, water and soil (18), (295) (296). The lack of data investigating the total indoor virome is likely due to a number of reasons. Viruses are very diverse and as such, there is no universal gene target analogous to the 16S rRNA gene found in bacteria and Archaea, making the simultaneous detection of multiple viruses costly and time-consuming. Also, once amplified, viral sequences must be compared to public databases such as BLAST. However, there are far fewer virus genomes in such databases than for other microorganisms and this can make identification difficult (294), (296). Also, viruses present in indoor locations will likely be in very low numbers, especially in non-outbreak situations and sampling methods may not be sufficient to capture their presence. The identification of RNA viruses and viruses with very small genomes also poses technical challenges. The concept of an 'indoor virome' may not be as relevant as the virome of other environmental samples and as such, previous work has focussed on determining the presence of specific viruses in indoor locations.

Routine microbiological screening does not often consider contamination with viruses and as previously mentioned, relies on the use of total viable counts of bacteria. Carducci et al. have investigated the use of the recently discovered torque teno virus (TTV) as a marker of viral environmental contamination (19). TTV has not, as yet, been associated with a specific disease state but has been isolated from

all bodily secretions and tissue types tested and has been reported to be present in over 90 % of the human population (297). Carducci et al. claim that despite a lack of data from indoor environments, microbial indicators currently used for environmental monitoring in the form of bacterial TVCs, do not provide a useful marker for viral presence and suggest that a viral marker may be more appropriate. Bacteria and viruses have different structures and properties and therefore it could be assumed that they have different survival characteristics in the environment.

5.4 Sampling for viruses

Sampling for viruses from the environment is problematic, this is particularly true of air sampling, as virions are easily damaged by most air samplers (5). Recovery of live virus from air and surface samples is not feasible if conducting a large sampling protocol or if checking a bed space is sufficiently decontaminated prior to a new patient admission. This is due to the need to grow virus in cell culture and often when recovering virus from environmental sources, the cultures are easily contaminated. Some clinically relevant viruses cannot currently be cultured *in vitro,* for example NV (20), (250). This means that molecular methods such as PCR are much more suited to the analysis of viruses in the environment.

5.4.1 Air sampling and analysis

Viruses are an important cause of RTIs (272), (298) and aerosol transmission is thought to play a role in the spread of many viruses of clinical significance (10),

(299). Non-respiratory viruses have also been isolated from the air (20), (250), (279) and the air may serve as a medium for spread of virus to surfaces where they can be picked up by hands and ingested, for example. The number of infections caused by inhalation of viral aerosols is not currently known (300).

There is no standard method available for the detection of virus bioaerosols (102), (299). Impinger and slit samplers (see Chapter 3) have been used when attempting to isolate viruses from the air as they may allow the collection of live, viable virus into a liquid medium (5), (299). However, these methods are not particularly suited for the detection of low virus concentrations partially due to the fact that to collect lower concentrations, the sampling time must be relatively long, which increases the risk of bacterial contamination. Also, these methods rely on the ability to culture captured viruses which is a time-consuming technique and some respiratory infections may be caused by viruses without optimised culturing protocols.

As with bacterial sampling, there has been a move towards the use of molecular techniques for environmental virus sampling. While the lack of demonstration of live, growing virus means that available molecular techniques cannot currently prove the presence of infectious virus, these techniques could indicate that if found in high quantities, at least a proportion might be infectious (21). A recent study showed significant positive correlations with the number of patients with upper and lower RTIs and the concentration of influenza A virus and AV isolated from the air. Enteroviruses were also isolated, although this was not correlated to clinical cases

(299). Molecular techniques do not require virions to remain undamaged through the sampling process as they only require the nucleic acid to be preserved.

5.5 Chapter aims

This was a proof-of-concept study, intended to determine if viral nucleic acids could be recovered from environmental surfaces and the air of the hospital being investigated. It was also to determine if bacterial TVCs could be used as an indicator for viral contamination and to investigate the use of torque teno virus (TTV) as an alternative marker.

5.6 Methods

5.6.1 Study design

Virus species were chosen based on the assumption that they may be present in a paediatric environment during the sampling period and for their clinical significance in this environment; NV, AV, RSV A, RSV B, RV and hMPV. CMV was included as a potential marker of mucosal contamination and as herpes viruses do not survive as long in the environment as AV, for example, CMV may provide a reference for recent contamination (301). TTV was included to investigate its use as a marker of contamination. A three-month screening protocol for the presence of these viruses was carried out.

Samples were taken from fixed high-touch sites and equivalent sites that have been shown to be contaminated with virus in previous studies. They were taken from a busy outpatient's waiting area during an infectious disease clinic and nephrology clinic (Clinic B) (Table 5.1 and Figure 5.2) once per month with a total of 78 samples being collected. Contact plates were also taken to determine bacterial TVCs.

Table 5.1: Virus screening sites in Clinic B outpatient's area

Sample Number	Sample Site	Sample Number	Sample Site
CB1	Chair	**CB14**	Toy cooker (bottom)
CB2	Heater	**CB15**	Plastic toy
CB3	Reception desk	**CB16**	Floor by toilet
CB4	Trolley top	**CB17**	Floor by room 9
CB5	Bin lid	**CB19**	Top of TV
CB6	Nurse desk	**H1**	Door handle WC
CB7	Nurse chair arm	**H2**	Door handle room 5
CB8	Chair arm	**H3**	Door handle room 6
CB9	Chair top	**H4**	Door handle room 7
CB10	Small table	**H5**	Door handle room 8
CB11	Large table	**H6**	Door handle room 9
CB12	Book	**H7**	Door handle room 10
CB13	Toy cooker (top)	**Air**	Nurse's station

The outpatient's area was chosen as it is small and self-contained with a high number of people attending on the clinic day chosen. Figure 5.3 shows the lay out of this area and Figure 5.4 shows close-ups of some of the areas sampled.

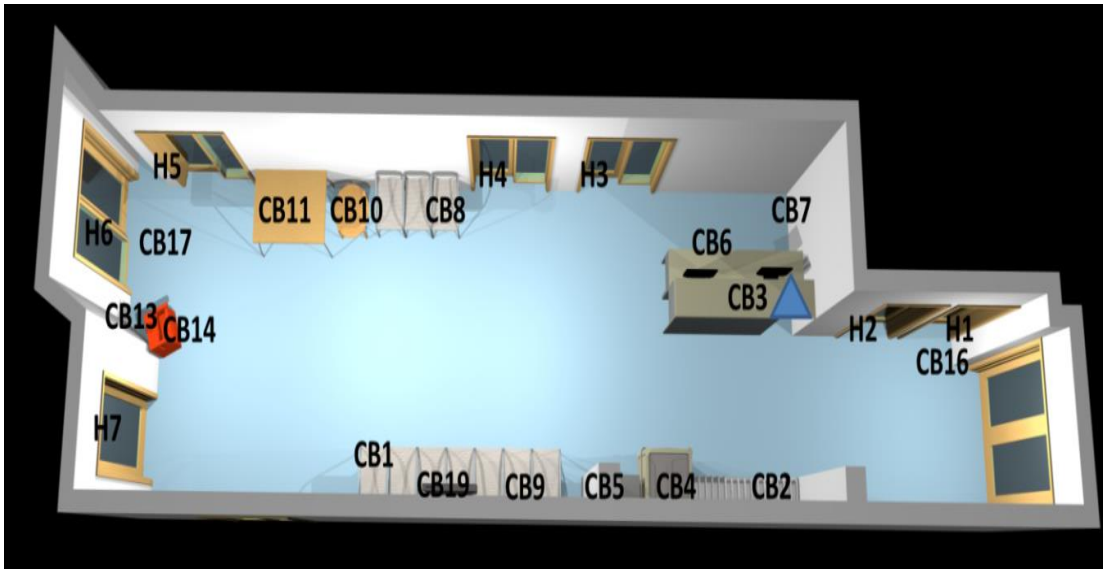


Figure 5.2: Diagram of Clinic B waiting area showing sampling sites. Air sampler is indicated by a blue triangle. CB18 is missing due to the site becoming unavailable for screening

Figure 5.3: Image of Clinic B outpatient's waiting area

Figure 5.4: Images showing sampling sites on Clinic B

5.6.2 Surface sampling

5.6.2.1 Swabbing

Surface swabbing was carried out as detailed in section 2.2.2.1 except with RNAlater stabilisation buffer (Qiagen, UK) as a wetting and storage solution.

5.6.2.2 Contact plates

TSA contact plates were taken using the method described in section 2.2.1.

5.6.3 Air sampling

Air sampling was carried out using a Burkard C90M cyclone sampler (Burkard, UK), placed on the nurse's station as indicated in Figure 4.3. The sampler was set at a flow rate of 16.6 l / min and left to run for 10 hours, starting 1 hour prior to the clinic start and ending 1 hour after the final patient vacated the area. Air samples were collected in 1 ml RNA*later* buffer (302).

5.6.4 Temperature and relative humidity

Temperature and RH were measured as described in section 2.2.4. HOBO samplers were left in three locations in the clinic; on the nurse's desk at the entrance to the area, on a trolley in the middle of the clinic and on top of the door frame to Room 10 at the back of the area.

5.6.5 Nucleic acid extraction

DNA and RNA were co-extracted using the AllPrep Mini Kit (Qiagen, UK). 10 µl of mouse cell IPC was added to serve as a DNA extraction and amplification control. 3 µl of phocine distemper virus (PDV) RNA was added to serve as an extraction and reverse transcription (RT) control. DNA and RNA were eluted in 50 µl of AE buffer or RNase-free water (Qiagen, UK) and processed immediately.

5.6.6 Virus qPCRs

qPCR was carried out according to in-house Standard Operating Procedures (SOPs) at GOSH. DNA assays were prepared by adding 12.5 µl QuantiFast Mastermix (Qiagen, UK) per reaction and RNA assays by adding 12.5 µl QuantiFast RT Mastermix and 0.2 µl reverse transcriptase enzyme (both Qiagen, UK) per reaction. Primer and probe mixes (MWG Eurofins, Germany) were added at a final concentration of 10 pmol / µl and cycling conditions carried out according to SOPs or Qiagen protocols. Table 5.2 shows primer and probe sequences.

Standard curves were made for AV and CMV as described in Chapter 2. Plasmid standards were provided by GOSH Virology department for RV, hMPV, NV GI and GII serotypes and RSV A and B. No standards were available for TTV. Samples were run on ABI 7500 Real-Time PCR system (Life Technologies, UK). DNA assay conditions were as follows; 1 cycle of 95 ^{O}C for 5 minutes followed by 45 cycles of 95 ^{O}C for 30 seconds and 60 ^{O}C for 30 seconds. RNA cycling consisted of 1 cycle of 50 ^{O}C for 20 minutes, one cycle of 95 ^{O}C for 5 minutes, followed by 45 cycles of 95 ^{O}C for 15 seconds and 60 ^{O}C for 30 seconds.

Amplification curves were viewed with ABI Sequence Detection Software (SDS) version 4.1. Results were plotted by SDS as fluorescence (nm) *vs*. number of qPCR cycles, with a threshold for detection of DNA-based fluorescence set just above background, as determined by the NTC. The cycle number at which the sample

fluorescence exceeded the background was recorded (C_t) and either compared to standards to provide a copy number or recorded as number of cycles taken to produce a positive result.

Table 5.2: Primer and probe sequences used for assessing viral contamination of surfaces and air in Clinic B

Target	Name	Sequence	5' reporter	3' quench
Adenovirus (303)	**Adeno UCL-F**	GCC ACS GTG GGG TTT CTA AAC TT		
	Adeno UCL-R	GCC CCA GTG GKC TTA CAT GCA CAT C		
	Adeno UCl-Pr	TGC ACC AGA CCC GGR CTC AGG TAC TCC GA	FAM	BHQ-1
CMV (304)	**CMV-F**	GCA TGC GCG AGT GTC AAG AC		
	CMV-R	GTT ACT TTG AGC GCC ATC TGT TCC T		
	CMV-Pr	TGC GCC GTA TGC TGC TCG ACA	JOE	BHQ-1
IPC (*Mus*)	**IPC (*Mus*)-F**	Unpublished, provided by Kathryn Harris at GOSH		
	IPC (*Mus*)-R	Unpublished, provided by Kathryn Harris at GOSH		
	IPC (*Mus*)-Pr	Unpublished, provided by Kathryn Harris at GOSH	CY5	BHQ-1
RSVA (305)	**RSVA-F**	AGA TCA ACT TCT GTC ATC CAG CAA		
	RSVA-R	TTC TGC ACA TCA TAA TTA GGA G		
	RSVA-Pr	CAC CAT CCA ACG GAG CAC AGG AGA T	CY5	BHQ-2
RSVB (305)	**RSVB-F**	AAG ATG CAA ATC ATA AAT TCA CAG GA		
	RSVB-R	TGA TAT CCA GCA TCT TTA AGT A		
	RSVB-Pr	TTT CCC TTC CTA ACC TGG ACAB TA	JOE	BHQ-1
hMPV	**HMPV1**	CAT ATA AGC ATG CTA TAT TAA AAG AGT CTC		
	HMPV2	CCT ATT TCT GCA GCA TAT TTG TAA TCA G		

Target	Name	Sequence	5' reporter	3' quench
	HMPV Pr	TGY AAT GAT GAG GGT GTC ACT GCG GTT G	FAM	BHQ-1
Norovirus	**COG-1F (GI F)**	CGY TGG ATG CGN TTY CAT GA		
	COG-1R (GI R)	CTT AGA CGC CAT CAT TYA C		
	RING 1a (GI Pr)	AGA TYG CGA TCY CCT GCT CA	FAM	BHQ-1
Norovirus (cont.)	**RING 1b (GI Pr)**	AGA TCG CGG TCT CCT GTC CA	FAM	BHQ-1
	QNIF 2 (GII F)	ATG TTC AGR TGG ATG AGR TTC TCW GA		
	COG-2R (GII R)	TGC ACG CCA TCT TCA TTC ACA		
	QNIFS (GII Pr)	AGC ACG TGG GAG GGC GAT CG	CY5	BHQ-2
Rotavirus	**ROTA F**	ACC ATC TWC ACR TRA CCC TCT ATG AG		
	ROTA R	GGT CAC ATA ACG CCC CTA TAG C		
	ROTA Pr	AGT TAA AAG CTA ACA CTG TCA AA	FAM	MGB
PDV	**PDV F**	GCG GGT GCC TTT TAC AAG AAC		
	PDV R	CAG AAT AAG CAA AAT TGA TAG GAA CCA T		
	PDV Pr	TCT TTC CTC AAC CTC GTC CGT CAC AGG T	CY3	BHQ-2

5.7 Results

5.7.1 Virus-related outbreaks and incidents at GOSH, 2012

The outbreaks and incidents of viral aetiology at GOSH for the year in which sampling took place can be seen in Table 5.3. NV was the leading cause of outbreaks throughout the sampling period.

Table 5.3: Virus-related outbreaks and incidents at GOSH 2012

Type of ward	Outbreak / incident	Pathogen	Patients involved	Staff involved	Action taken
Neurosurgery	D&V[a]	Unknown	4	1	L3[b] clean
Cardiac	Un-reported chickenpox	VZV	1	0	Contacts immunised
Neurology HDU[c]	D&V	Unknown	4	8	L3 clean, restricted ward access
Cardiac-Respiratory	D&V	NV, RV	14, 5	Many	L3 clean, restricted ward access
Neurology HDU	D&V	Unknown	6	9	Isolation, L3 clean
Neurology HDU	D&V	NV	9	0	Isolation, restricted access, L3 clean
ICT	D&V	NV	0	5	L3 clean
BMT[d] & HOU[e]	D&V	NV	3		L3 Clean
BMT	Screening failure	AV	-	-	L3 clean
BMT	D&V	NV	5	1	Restricted access, L3 clean
Cardiac	Rash	VZV	4	0	Contact screen
BMT & HOU	Un-notified	VZV	1	0	Contact

Outpatients	chickenpox				screen
General surgery	Chickenpox	VZV	2	0	Immunisation
Respiratory	D&V	NV	4	0	L3 clean of sluice room
Spinal surgery	Chickenpox	VZV	4	0	Contact screen
Dermatology	Chickenpox	VZV	1	0	Contact screen
Investigative	Chickenpox – failure to notify	VZV	2	0	Immunisation
HOU	Influenza	Influenza A	4	0	Communal area closed restricted access.
Cardiac	Para-influenza	Para-influenza	3	0	Ward closure
Neurology	D&V	NV	7	0	Ward closure
Nephrology	D&V	NV	3	5	Restricted access, L3 clean
Nephrology	Chickenpox	VZV	1	0	Contact screen
Neurology HDU	Shingles	VZV	0	1	Contact screen
Metabolic disorders	D&V	NV	1	11	Restricted access, L3 clean
Respiratory	D&V	NV	3	1	L3 clean
Immunology	Screening failure	AV	0	0	L3 clean
Investigative	Chickenpox	VZV	1	0	Contact screen
Rheumology	D&V	NV	1	0	L3 clean
BMT	D&V	NV	4	9	Restricted access, L3 clean
BMT, HOU	Screening failure	AV	0	0	L3 clean

a) Diarrhoea and vomiting b) Level 3 clean (chlorine, intensive) c) High-dependency unit

d) Bone marrow transplant e) Haematology and Oncology

5.7.2 Standards and controls for virus qPCRs

All standards and controls were within GOSH SOP recommended parameters. IPC and PDV controls were within ranges used by GOSH Virology to ensure that the samples were not inhibited. Any sample with a DNA IPC of > 30 C_t was repeated at a 1:10 dilution. Any sample with an RNA IPC (PDV) C_t of > 30 was repeated at a 1:10 dilution. All NTCs were negative.

5.7.3 Temperature and relative humidity

The average temperature for the November sampling period was 23 ^{O}C with an average RH of 27 %. For December, the average temperature was slightly higher at 26 ^{O}C and RH a little lower at 25 %. Large differences were observed in January, with an average temperature of 18 ^{O}C and RH of 53 %

5.7.4 Bacterial total viable counts

CFUs recovered from each site at each time point can be seen in Table 5.4. Table cells are colour coded to provide an indication of low, intermediate, high and very high bacterial contamination. The majority of sites sampled had < 50 CFU across all three time points. The top of the toy cooker (CB13) had 0 CFU at all time points, the bottom of the toy cooker (CB14), plastic toy (CB15) and trolley top (CB4) all had 0 or very low CFUs also. The chair arm (CB8) had high levels of contamination at all time points. The top of the TV (CB19) in January was the only site to have very high

CFUs at any time point but the reception desk (CB3) and chair (CB1) each had high levels at one or more time points.

Table 5.4: Total bacterial counts recovered from Clinic B sampling sites over 3 time points

		CFU Count		
Sample	**Site**	**November**	**December**	**January**
CB1	Chair	26	109	64
CB2	Heater	22	24	21
CB3	Reception desk	109	1	44
CB4	Trolley top	2	0	0
CB5	Bin	70	4	56
CB6	Nurse desk	2	9	34
CB7	Nurse chair arm	33	1	3
CB8	Chair arm	153	152	106
CB9	Chair top	119	56	142
CB10	Small table	39	56	1
CB11	Big table	0	1	0
CB12	Book	14	38	40
CB13	Toy cooker (top)	0	0	0
CB14	Toy cooker (bottom)	0	1	0
CB15	Plastic toy	1	0	0
CB16	Floor by WC	72	36	99
CB17	Floor room 9	38	56	53
CB19	Top of TV	56	60	300

Green: < 50 CFU, Yellow: 50–100 CFU, Orange: 101–200 CFU, Red: > 200 CFU

5.7.5 Virus qPCR results

5.7.5.1 Adenovirus

The AV qPCR C_t results are shown in Table 5.5. AV was found throughout the clinic waiting area for all months sampled. Of 78 samples taken, 29 (37 %) were positive

for the presence of AV DNA. More sites were positive in November (38 %) and December (61 %) than in January (11 %). AV DNA was only isolated from the air at the November time point.

Table 5.6 shows C_t values translated into copy number of AV for positive sites. Copy number per ml of sample, as reported, is only semi-quantitative as it assumes all virus particles were released from the cotton swab. This is unlikely to be the case and as such, the figures are possibly an underestimate of virus copy number at each site. The highest copy number was recovered from the top of the TV (CB19) in November (20625 copies / ml). Lower numbers (but still relatively high) were found on top of the chair (CB9) (7711 copies / ml) and the nurse's desk (CB7) and chair arm (CB8). Similar numbers of around 4000 copies / ml were recovered from 2 door handles, the nurse's desk and the bin in December and overall copy numbers were lower in January.

Table 5.5: Adenovirus detection by qPCR over three months obtained from outpatient's clinic at GOSH.

		C_t		
Sample	**Site**	**November**	**December**	**January**
CB1	Chair	Undetermined	40.53	Undetermined
CB2	Heater	Undetermined	38.7	Undetermined
CB3	Reception desk	30.601	34.96	Undetermined
CB4	Trolley top	Undetermined	33.54	Undetermined
CB5	Bin lid	Undetermined	34.88	40.14
CB6	Nurse desk	Undetermined	35.27	Undetermined
CB7	Nurse chair arm	30.53	36.52	Undetermined
CB8	Chair arm	Undetermined	Undetermined	Undetermined
CB9	Top of chair	31.33	39.25	Undetermined
CB10	Small table	Undetermined	Undetermined	Undetermined
CB11	Large table	Undetermined	Undetermined	41.48
CB12	Book	Undetermined	39.6	Undetermined
CB13	Toy cooker (top)	41.79	42.29	Undetermined
CB14	Toy cooker (bottom)	Undetermined	39.98	Undetermined
CB15	Plastic toy	37.67	Undetermined	Undetermined
CB16	Floor by WC	34.73	Undetermined	Undetermined
CB17	Floor by room 9	Undetermined	Undetermined	Undetermined
CB19	Top of TV	31.39	Undetermined	Undetermined
H1	Handle to WC	Undetermined	38	Undetermined
H2	Handle room 5	Undetermined	34.67	Undetermined
H3	Handle room 6	43.48	37.14	Undetermined
H4	Handle room 7	Undetermined	34.25	Undetermined
H5	Handle room 8	Undetermined	36.48	Undetermined
H6	Handle room 9	43.19	Undetermined	Undetermined
H7	Handle room 10	Undetermined	Undetermined	44.23
Air	Air	40.5	Undetermined	Undetermined

Table 5.6: Adenovirus copy number at each positive site over three month sampling period of an outpatient's area at GOSH.

Sample	Site	Copies / ml
CB3 Nov	Reception desk	1450
CB7 Nov	Nurse chair arm	1255
CB9 Nov	Top of chair	7711
CB13 Nov	Toy cooker (top)	4
CB15 Nov	Plastic toy	205
CB16 Nov	Floor by WC	344
CB19 Nov	Top of TV	20625
H3 Nov	Handle room 6	2
H6 Nov	Handle room 9	4
Air Nov	Air	6
CB1 Dec	Chair	2
CB2 Dec	Heater	10
CB3 Dec	Reception desk	4072
CB4 Dec	Trolley top	8281
CB5 Dec	Bin lid	4023
CB6 Dec	Nurse desk	349
CB7 Dec	Nurse chair arm	30
CB9 Dec	Top of chair	7
CB12 Dec	Book	8
CB13 Dec	Toy cooker (top)	4
CB14 Dec	Toy cooker (bottom)	8
H1 Dec	Handle to WC	12
H2 Dec	Handle room 5	4079
H3 Dec	Handle room 6	14
H4 Dec	Handle room 7	4057
H5 Dec	Handle room 8	31
CB5 Jan	Bin lid	26
CB11 Jan	Large table	9
H7 Jan	Handle room 10	1

5.7.5.2 CMV

Of 78 sites swabbed, 5 were positive for the presence of CMV DNA (6 %). These are shown in Table 5.7 along with copy number values as determined by comparing to the standard curve of known copy number. CMV was isolated from the air during the December time point and the highest copy number recovered was from the heater in November.

Table 5.7: Cytomegalovirus positive sites over three month sampling period of outpatient's area, showing C_t value and copy number / ml.

Sample	Site	C_t	Copies / ml
CB2 Nov	Heater	36.53	296
CB19 Nov	Top of TV	41.50	7
CB17 Dec	Floor by room 9	37.27	169
CB Air Dec	Air	37.65	128
CB11 Jan	Large table	38.74	56

5.7.5.3 Rotavirus

Two door handles were positive for the presence of RV nucleic acid; the door handle to the WC (H1 Nov) and the door handle to Consultant room 5 (H2 Nov). They showed C_ts of 38.30 and 41.19 respectively. These can be seen on the amplification plot in Figure 5.5.

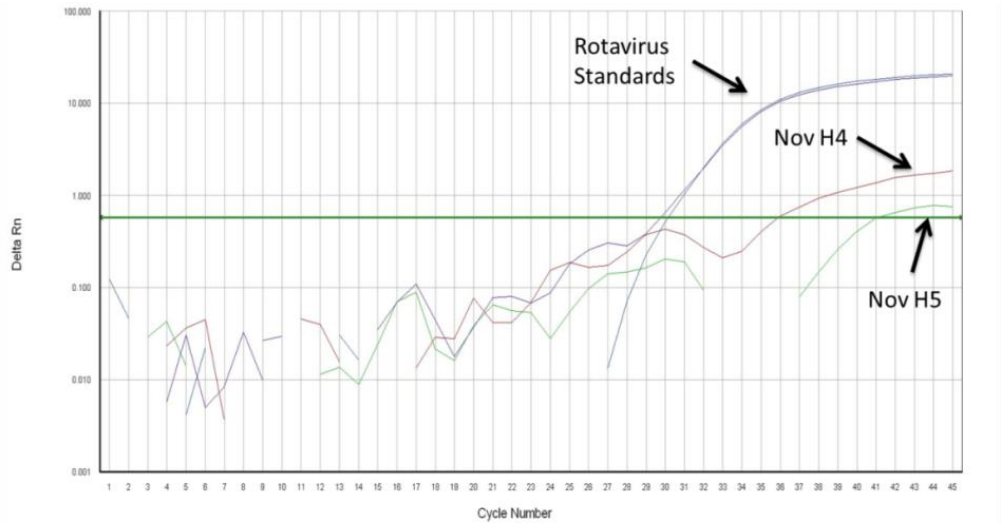


Figure 5.5: Fluorescence *vs*. Cycle plot showing Rotavirus standards and positive sites from outpatient's clinic

5.7.5.4 Norovirus

One site had the presence of NV nucleic acid, the November 16 swab taken from the floor outside the toilet. This sample was positive for NV serotype GII and had a C_t of 38.40 (Figure 5.6). No samples were positive for NV GI serotype.

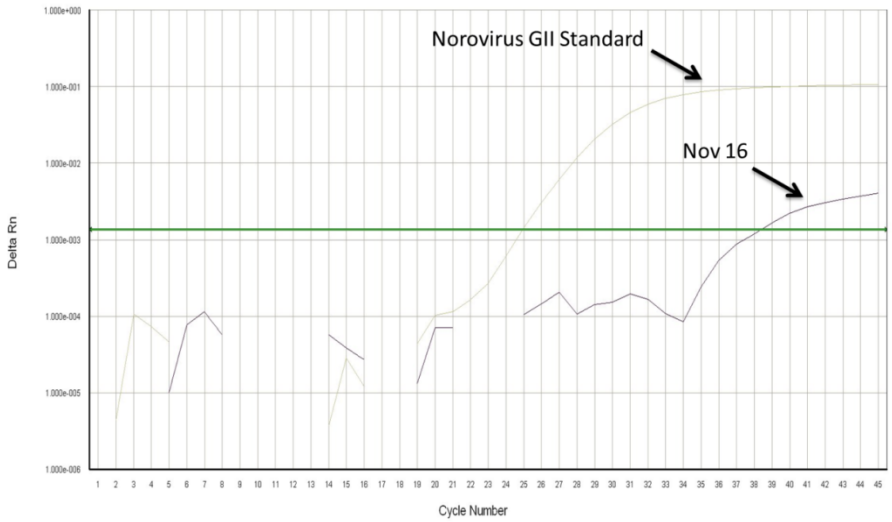


Figure 5.6: Fluorescence vs. Cycle plot showing Norovirus GII standard and positive sample from outpatient's clinic

5.7.5.5 Respiratory Syncytial Virus and Human Metapneumovirus

Of 78 samples taken over the three month period, none were positive for the presence of RSV A, RSV B or hMPV nucleic acid.

5.7.5.8 Torque Teno Virus

Fifteen sites sampled were positive for the presence of TTV DNA (19 %). The TTV virus results are shown in Table 5.8. There were 5 TTV positive sites in November, 6 in December and 4 in January. The book (CB12) was positive at all 3 time points. The heater (CB2) and plastic toy (CB15) were positive at the November and December time points but not at the January sampling. As no standard curves were constructed for TTV, copy numbers could not be calculated.

Table 5.8: Torque teno virus positive sites over three month sampling period of outpatient's area, showing C_t values

Sample	Site	C_t
CB2 Nov	Heater	37.62
CB12 Nov	Book	40.1
CB15 Nov	Plastic toy	37.4
CB16 Nov	Floor by WC	40
H2 Nov	Handle room 5	40.76
CB2 Dec	Heater	39.66
CB10 Dec	Small table	36.8
CB12 Dec	Book	38.87
CB14 Dec	Toy cooker (bottom)	42.38
CB15 Dec	Plastic toy	39.4
H1 Dec	Handle WC	39.4
CB1 Jan	Chair	36.73
CB5 Jan	Bin lid	39.37
CB11 Jan	Large table	37.61
CB12 Jan	Book	38

5.7.5.9 Collated results

Figures 5.7 – 5.9 show schematics of the clinic area for each month with indicators of TVCs and sites from which virus was recovered. Air samples are not included. January had the least overall virus recovery whilst November and December were similar for the numbers of virus positive sites. Virus was recovered from a range of sites across the clinic area and CFUs were below 200 at all time points, except the top of the TV in January. Virus nucleic acid was recovered from sites with a range of TVC counts.

Figure 5.7: Diagram of clinic area showing bacterial total viable counts and virus positive sites: November. A= Adenovirus, T= Torque teno virus, R = Rotavirus, C=Cytomegalovirus, N=Norovirus

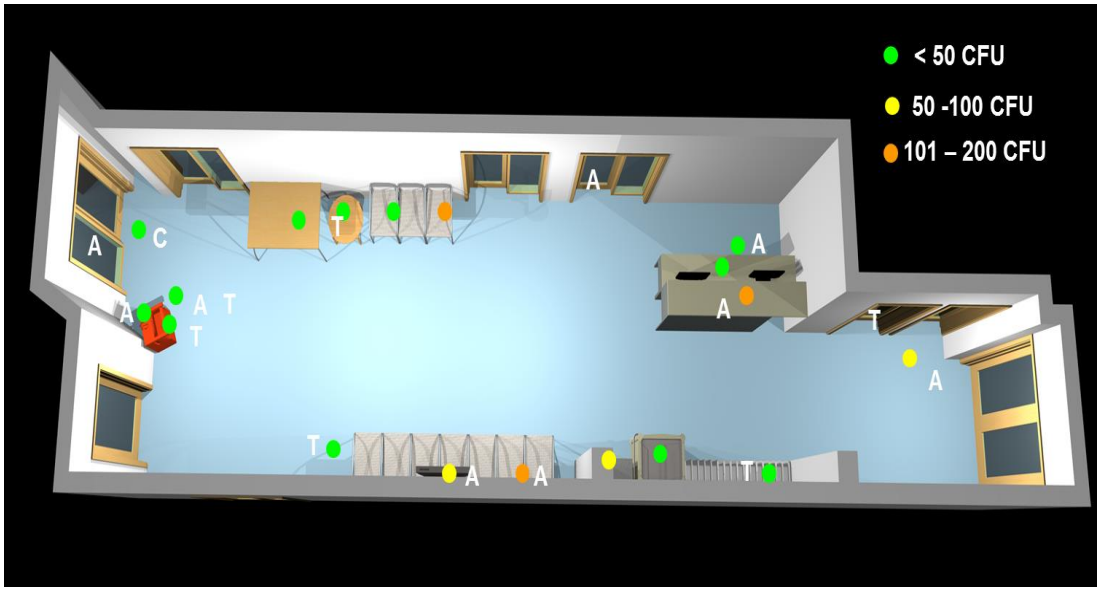


Figure 5.8: Diagram of clinic area showing bacterial total viable counts and virus positive sites: December A= Adenovirus, T= Torque teno virus, R = Rotavirus, C=Cytomegalovirus

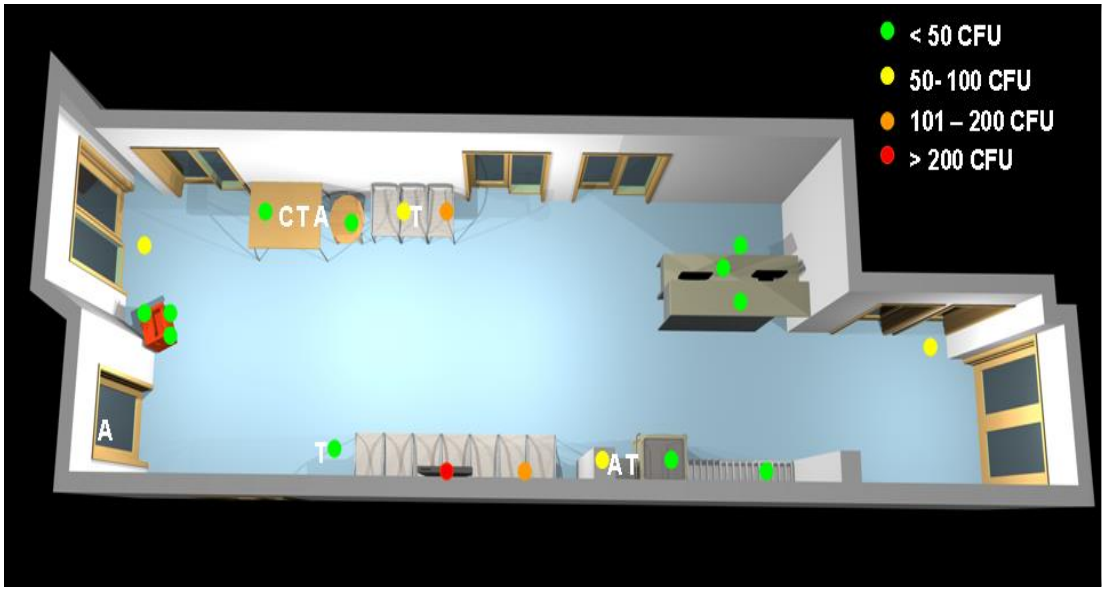


Figure 5.9: Diagram of clinic area showing bacterial total viable counts and virus positive sites: January. A= Adenovirus, T= Torque teno virus, C=Cytomegalovirus

5.8 Discussion

A three month sampling protocol to detect viral nucleic acids was carried out in an infectious disease and nephrology clinic outpatient's waiting area. Virus nucleic acid was found on a variety of surfaces and in the air. Of all sites sampled 46 % were positive for the presence of viral nucleic acids. Of these, 26 % were positive for more than one virus, with AV and TTV most commonly being identified together at the same location.

Viruses have been detected by other researchers in similar environments and are a common cause of indoor-acquired infectious disease (306). Gallimore et al.

demonstrated the presence of NV nucleic acid in 18 % of swabs, RV in 14 % and Astrovirus (AsV) in 4 % of swabs on a UK children's ward, despite correct infection control and cleaning procedures being in place. Ganime et al. also detected RV on 14 % of environmental sites sampled, finding between 3.4 and 2.9 x 10^3 copies / ml (307). A recent study by Pankhurst et al. (under review) reported extensive environmental contamination in hospital cubicles and detected NV at similar rates to the current work. The environmental presence of nucleic acid from viral pathogens is clearly a universal issue. Differences in species identified depend on detection assays performed but may also reflect unique regional viromes.

Correlating environmental contamination with risk is difficult as infectious doses are often hard to determine for a number of viruses. They depend on a number of complex factors including host-related elements such as immune status. However, one study reported that only 6.6 AV type 4 viral particles were required to infect 50 % of a test population via the aerosol route (308). For clinical diagnosis of AV infection at GOSH, any patient sample with a C_t of 40 or below is deemed as positive. The copy numbers of AV found in the current study were often far in excess of 6.6 and C_ts were largely less than 40. Although no direct link can be made to AV DNA copy numbers on surfaces and infectious dose, it might be suggested that levels as high as were observed might pose a risk to the susceptible patient.

NV was the leading cause of known viral outbreaks at GOSH during the sampling period (Table 5.3). This is likely to reflect data from other trusts but the actual case

number remains relatively low due to the ability to isolate patients quickly at GOSH. However, the virus does spread rapidly and it is often not possible to prevent it from passing from ward to ward. NV outbreaks at GOSH do not tend to follow the seasonal trend as observed in other hospitals and outbreaks occur throughout the year. This is due in part to the large numbers of immunocompromised children present in the hospital. These children will shed virus for a long period of time whilst possibly remaining asymptomatic, causing contamination of the environment and spread of disease. NV RNA was found in the outpatient's clinic waiting area but no outbreaks were reported in the area.

Figure 5.10 shows the number of cases of diarrhoea and vomiting (D&V) at GOSH during 2012. NV was the most commonly isolated organism during these outbreaks; however in some cases RV was the causative agent. Patients with D&V associated with known *C. difficile* or other bacterial aetiology have not been included in this figure. It can be seen that cases occurred throughout the year but with a large peak in February.

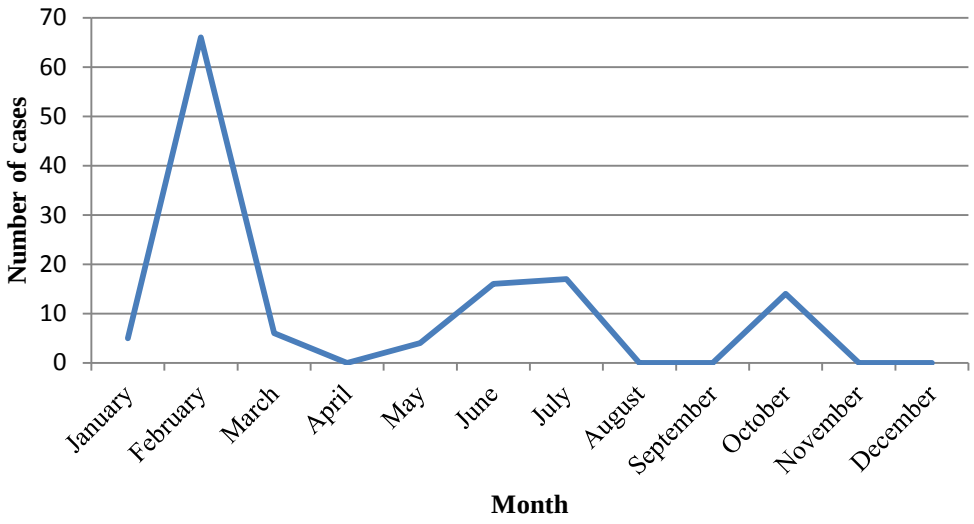


Figure 5.10: Diarrhoea and vomiting cases at GOSH during 2012

5.8.1 Sites most frequently contaminated

Handles were frequently contaminated in this study and this is in agreement with previous work carried out in other areas of GOSH (Pankhurst et al., under review). The common contamination of door handles with bacteria was recently investigated by Wojgani et al. They found frequent contamination of door handles within healthcare environments and that use of a handle was correlated to levels of contamination (p= < 0.01) (44). Bacterial load on door handles was not investigated during the study presented in this thesis as the whole of each handle was swabbed for the identification of virus. However, the current findings suggest that bacteria may follow a similar contamination pattern. The two clinics have approximately 30 patients per day and all enter Consultant's rooms through doors which must be opened using the handle. Viruses may have been directly deposited from the air or

via hands from a person infected with the virus or via another object within the clinic area.

5.8.2 Frequency of contamination

Overall, with the exception of AV, viruses were found with relatively low frequency, especially when compared to TVCs for bacteria in the same sampling period; 80 % of sites sampled were positive for bacterial growth. Unlike bacteria, viruses cannot replicate outside of a host and as such will not multiply in the inanimate environment. They are subject to degradation by various mechanisms including temperature fluctuations, desiccation and exposure to UV-light but viral nucleic acids can persist for prolonged periods of time on surfaces. The isolation of viral nucleic acids from the environment indicates that virus was present and even low C_t values could translate to copy numbers that might pose a risk for a number of viruses investigated.

The potential presence of children who may have had asymptomatic NV or RV infection and who may have had a high proportion of respiratory viral infections, especially during the winter months, would perhaps have led to the expectation that the rates of recovery of viral nucleic acid might be higher. The lack of more sites contaminated with those viruses associated with acute disease (hMPV, NV, RV and RSV) could be due to the fact that the clinic area is for children and their families awaiting outpatient appointments. They would be less likely to attend an appointment if the child had acute clinical symptoms, such as diarrhoea or vomiting

in the case of RV or NV or were unwell with respiratory symptoms. Low numbers of virus positive sites could also be due to the number of sites sampled. A range of sites and all of those that could be accessed were swabbed but they still equate to only a small area within the whole clinic. By increasing the swab area, more sites may have shown viral contamination. It must also be borne in mind that not all viruses present on a surface may be picked up by the swabbing method used and not all viruses on the swab would be released into solution. In Chapter 3, it was demonstrated that > 10 % of bacteria were recovered from a test surface using swabs. Whilst viruses are smaller and may not be trapped so easily in the swab, it might be expected that virus recovery is also not complete, leading to an underestimation of virus present.

Viruses may have been transported into the area by healthcare workers or visitors from another part of the hospital or even from an external location. Without screening patients and staff and carrying out molecular typing, it is impossible to know what the source of the viral contamination is, however the presence of these viruses in a clinical environment is of concern for patients and staff alike.

5.8.3 Air sampling

AV and CMV nucleic acid was found in air samples taken from the clinic. AV was isolated in November and CMV in December. There are many incidences of AV being isolated from the indoor air (19), (259), (299), (309) but only one study was identified that isolated CMV from the air. Mcluskey et al. isolated CMV from the air of 3 rooms, two housing immunocompromised patients with active CMV infection

and one with a patient with latent CMV infection (310). The lack of data regarding the isolation of CMV from the air might be as a result of CMV not being considered an 'airborne' virus and not being associated with nosocomial outbreaks on the same scale as NV, for example.

5.8.4 Markers for viral contamination

Carducci et al. have suggested the identification of TTV nucleic acid as a marker of environmental virus contamination as opposed to relying on bacterial counts (19). Viral nucleic acid was found in 29 of 176 samples during their screening, with the majority of those being positive for only TTV (10 %) and only TTV DNA was found in air samples (19). The rates of NV and AV recovery were lower than previous studies and those found in Clinic B in the current study. This could be due to local differences or differences in carriage rates in the people present at or near the sampling time. Carducci et al. concluded that TTV could be used more reliably as a marker but no other data are as yet available to compare their findings to. The use of TTV as a marker for viral contamination in drinking water has however also been proposed (311).

The current study found TTV in 11.7 % of samples taken, suggesting that similar levels can be found in different indoor environments. Carducci et al. found no sites positive for RV compared with 1.6 % in the current study and only 0.8 % of sites positive for AV compared to 37 % but both studies found one site contaminated with NV (0.8 %). The results between the two studies are remarkably similar (apart from

for AV) and perhaps suggest that TTV is present in a variety of indoor environments and could indicate cleaning failures, suggesting the possibility that other viruses may be present. TTV has been found in exhaled breath (312) and various other bodily secretions and has been found in over 90 % people tested regardless of age, sex or race (297) so it may be expected that it would be found in high levels in indoor environments. However, as has been outlined in the current chapter, viruses are very varied in their abilities to survive in the environment and survival can be dependent on physical parameters such as humidity and temperature. Due to the lack of experimental survival data for TTV, it cannot be assumed that it behaves in the same way as viral pathogens. At all time points, nucleic acid from potentially pathogenic viruses was identified from sites that were negative for the presence of TTV. This suggests that if TTV is to be recommended as a marker, it must be taken into account that a variety of sites must be screened in order to ensure detection. TTV may however provide a better indication in a clinical environment for viral contamination than the use of bacterial markers. In the current study, virus was identified on a number of items which showed no or very little bacterial growth.

5.8.5 Temperature and RH

Temperature and relative RH are among the most commonly studied parameters when looking at virus stability in the environment (288). Abad et al. found that resistance to desiccation was one of the most important factors in determining environmental survival of virus (280). They also found the presence of faecal matter prolonged survival of poliovirus (PV) and AV but not of RV and that hepatitis, PV

and AV all survived better at 4 ^{O}C than RV. In their study, AV survival was not influenced by RH which is in contrast to other work showing that AV is more stable at high RH (313). In another study AV survived best at a medium RH of 50 % (87). Absolute humidity has also been shown to be more important in virus survival than RH, with low humidity often being a predictor of influenza A outbreaks (313).

Irregular temperature and RH readings for January could be a reason for the lower number of virus-positive sites at that time point. Information regarding AV stability at different RH is conflicting with some reporting stability at high RH (280) and some at medium RH (297). The current study shows the presence of AV at lower RH of 25 and 27 % and a reduction in positive sites at a medium RH of 53 %. Different viruses behave differently at different temperatures and RH and in different studies; therefore it is difficult to know if these factors were the reason for lower numbers of positive sites in January. They could however, have been contributing factors. Methods of recovery are not always comparable across studies by different authors and further investigation specifically taking into account physical parameters is required in order for any firm conclusions to be made.

5.9 Conclusion

This study strengthens previous work carried out at GOSH and in other healthcare facilities, adding to the evidence that nucleic acids of pathogenic viruses can be found in hospital environments and may be indicative of infectious virus being present. The location sampled was not an area attended by patients at particular risk

but they often travelled to other areas of the hospital, as did doctors, consultants, and other staff and therefore, could have taken pathogenic viruses with them to more vulnerable patients. It has been suggested that in high-risk environments such as hospitals and with the ease and availability of molecular techniques, viruses should be included in routine infection control monitoring and the current study adds evidence to support that case. Virus transmission in the indoor environment is complex and is dependent on a number of factors including host immunity, infectious dose, survival times on fomites and physical parameters. Surfaces and air undeniably contribute to the spread of viral infectious diseases and should be further investigated to determine their exact role for individual pathogens.

6. Microbial life on a hospital ward

6.1 Introduction

The environment has been recognised as a source of pathogen dissemination in a number of hospital studies (14), (40), (66), (73), (86), (174), (314), (315), (316), (317). Transmission via the air has been demonstrated for clinically significant microorganisms, for example MRSA from skin shedding or the nasal passages (318), *P. aeruginosa* from water sources (66) and shedding from cystic fibrosis (CF) patients (40) . The airborne dispersal of *C. difficile* has also been reported in hospital environments and the organism has been found on items in patient bed spaces such as tables, bins and sinks (174). It has been suggested that approximately 10-20 % of HCAIs occur as a result of exposure to airborne microorganisms (319) and a recent study by Bernard et al. detected multidrug-resistant bacteria in the air of 66 % of tested hospital sites (14). Despite the implications of hospital outbreaks, there is no standard procedure for the routine monitoring of microbial contamination in UK healthcare environments (46). There have also been no studies concerned with the systematic, long-term analysis of sources and development of microbial communities in hospitals. There have been studies carried out calculating total microbial counts in hospital rooms (46) and the detection of bacteria through surface swabbing is

common, yet methods vary widely across the literature and still largely rely on culture techniques.

There is a tendency to only consider the environment after an outbreak has begun. Environmental samples are collected in order to attempt to identify the outbreak source and measures are taken to prevent the incident happening again. There have been many published cases of this, for example environmental swabbing lead to a *K. pneumoniae* outbreak being linked to a sink that was being used as both a clean water source and for waste disposal in a report by Starlander and Melhus (33). Another study by Barbolla et al. found *A. baumanii* in the environment to be the same strain as was colonising and infecting patients in an intensive-care unit (ICU) (175). Multi-drug resistant *P. aeruginosa* was detected in sinks and tap water and proposed as the source of 2 outbreaks in different hospitals in South England, one of which reported a 40 % case fatality rate (66). Drug- resistant *P. aeruginosa* was also found in high levels in the air and on surfaces by Ferroni et al. when screening CF patient rooms (40). A great deal of research regarding microorganisms in hospitals and the role of the environment appears in the past, to have been concerned with bacterial and fungal counts, generally from the air. For example, a year-long study was carried out in Poland investigating the seasonal variability in airborne microbial counts by Augustowska et al. (38). They found statistically significant differences in the number of bacteria across the seasons but failed to identify what they had isolated. This also extends to the contamination of surfaces adjacent to patients who are mechanically ventilated (320).

Another topic that is of interest in hospitals is the contamination of patient bed spaces, focusing on a pathogen of interest. A large-scale study was carried out in a UK teaching hospital assessing the role of the environment in the transmission of MRSA (321). Environmental swabs were taken from bed areas and from telephones and also healthcare workers hands. It was found that MRSA-colonised patients contaminated their surroundings but in this case, this was not linked to further transmission to other patients. This is contrary to other reports which suggest that a patient is around 70 % more likely to acquire *S. aureus*, VRE, *P. aeruginosa*, *A. baumanii* and *C. difficile* when admitted to a room that was previously occupied by an infected or colonised patient (162), (106). Petti et al. also claim the environment is key in the transmission and spread of MRSA and found that despite only detecting low levels in the hospital environment, the risk of acquiring the bacteria was high due to its long survival times (73). Other studies tend to look at specific equipment such as computer keyboards (322) or stethoscopes (323), for example. The above cases and others from the literature focus on contamination of hospital environments from an infection control perspective. As a result, microbial communities are generally not fully characterised (324).

Despite the wealth of information regarding specific pathogens and evidence for the environment as a key factor in the transmission of HCAIs, the rates of infection remain high and with factors such as increasing drug-resistance, risk to patients is ever-increasing. As previously discussed, the most common source of microorganisms in an indoor environment is the presence of humans. In recent

research using NGS technologies, it has been found that humans can alter the microbiome of homes within 4-6 days (63). Therefore, the opportunity to analyse the hospital environment prior to use and occupation to determine how and when the area becomes colonised by microorganisms is of great interest and value. The information gained regarding what bacterial taxa are present, if they have a specific niche and what their origins are could have important implications for infection control, and cleaning practices.

Currently, culture-based screening is still used widely to monitor bacterial contamination within healthcare settings. It is a rapid, cheap method to indicate potential cleaning failures but cannot always be indicative of risk. However, this method can still provide important information. Long-term monitoring of bioburden may allow the discovery of problem areas, times of the year, or specific conditions that point to higher levels of contamination.

Using a combination of culture and NGS technologies to investigate a hospital ward on a long-term basis will provide important information with a variety of applications. Culture methods in the form of total viable counts have been discussed elsewhere and an overview of NGS is given below.

6.1.1 DNA sequencing

DNA sequencing was developed in the 1970's (120) and over the last 4 decades, has been widely used as powerful tool for genetic analysis. The original method, now

known as Sanger sequencing, became automated in 1987 and involves the use of modified, fluorescently-labelled nucleotides to provide sequence information. The wide-ranging implications of this technology inarguably peaked in 2001 with the sequencing of the human genome, where over the course of 13 years, the whole human 'genetic code' was mapped [151], [152].

Despite its accuracy and continuing relevance, Sanger sequencing has numerous limitations; it is labour-intensive, relatively expensive and due to the need for a cloning step when analysing microbial communities, is subject to biases such as reduced ability to clone AT-rich regions (327). The method does however produce a long read length (the number of unbroken sequenced bases) of 1000-1200 base pairs (bp). This is good for bacterial identification purposes, especially when considering the 16S rRNA gene which is approximately 1500 bp long (328). Despite remaining the gold-standard for sequencing in many laboratories and clinical environments, the cost and labour-intensive processes involved in Sanger sequencing have driven the development of faster and cheaper methods over recent years.

6.1.2 Next-generation sequencing

The new wave of sequencing technologies has become known as 'next–generation sequencing'. NGS allows the sequencing of millions of fragments of DNA simultaneously in a process known as massively-parallel sequencing (MPS). Megabases (Mb) to gigabases (Gb) of sequence reads can be produced in a single run. MPS allows high-throughput applications to be achieved, meaning that a whole

microbial genome can currently be sequenced in a matter of days (329). At the time of writing, five main sequencing strategies were available in the form of automated platforms; pyrosequencing, sequencing-by-synthesis, sequencing-by-ligation, ion semi-conductor technology and single molecule real-time sequencing (SMRT). Each platform and its associated chemistries are constantly updated in order to provide the most information in the quickest time, for the lowest cost. This has largely been driven by the race to sequence a human genome for $1000 but bench-top sequencers including the Roche GS Junior and the Illumina MiSeq have also been developed allowing much wider access to NGS technologies.

With the right platform, NGS allows the study of complex microbial communities which have been sampled directly from their environment, without culturing and their subsequent analysis in a single sequencing run. The depth of sequencing is so great that less abundant, rare species can be identified in a complex sample, provided that data analysis is performed correctly. NGS has been applied to a variety of research topics, including investigating the microbial component of various health conditions. For example, Blainey et al. reported a significant difference in lung microbial communities between healthy and CF patients, with that of healthy people being more diverse (330). The lung was previously thought to be a sterile site but NGS has proven this not to be the case (331), (332).

NGS is also applicable to the study of microbial communities by 16S rDNA sequence analysis from complex pools. The pioneering work using NGS for this

application was conducted by Caporaso et al., who in 2010, published the first protocol for microbial community analysis on an Illumina platform using custom primer chemistry (145). They then went on to publish the first protocol for using the Illumina MiSeq in 2012 and determined that the scaled down version of previous technology could accurately and reproducibly identify differences in microbial communities and was suitable for routine environmental monitoring (226).

Table 6.1 Shows a selection of the main NGS sequencing technologies available at the time of writing and gives examples of some commercially available platforms using each technology. The type of sequencing carried out, read length, data output and average cost per sequencing run of various chemistries are given as is that of an ABI 3730 Sanger sequencer for comparison. Study type and funding are likely to be key factors when selecting a platform as costs, throughput and output vary greatly.

Long reads tend to be preferable when conducting whole genome sequencing for example but lower cost, shorter reads are applicable for gene expression or community studies (333).

Table 6.1: Comparison of commercially available next-generation sequencing platforms.

Method	Template preparation	Platform examples	Bases / read	Run time	Millions of reads / run	Yield MB / run	Reagent cost / run (£)	Reagent cost/MB (£)
Pyrosequencing (454)	Clonal em-PCR	Roche GS FLX Titanium XL+	700	23 hours	1	700	3850	5.50
		Roche GS FLX Titanium XLR70	450	10 hours	1	450	2930	6.51
		Roche 454 GS Junior. Titanium	400	10 hours	0.1	50	683	13.66
Sequencing-by-synthesis	Clonal bridge amplification	Illumina HiSeq 2500	2 x 150	40 hours	600	180000	3816	0.02
		Illumina MiSeq v.2	2 x 250	39 hours	15	7500	700	0.09
		Illumina MiSeq v.3	2 x 300	~65 hours	25	15360	947	0.06
Sequencing-by-ligation (SOLiD)	Clonal em-PCR	Life Technologies SOLiD 5500 W Series V2.0	75 + 53	8.5 days	1410	155100	2050	0.01
Ion semiconductor (synthesis)	Clonal em-PCR	Life Technologies Ion PGM 318	400	7.3 hours	4	1500	459	0.31
		Ion Torrent 314 chip	400	3.7 hours	0.1	60 - 100	335	3.94
Single molecule real-time synthesis	Single molecule (no amplification)	PacBio RS II	3000	< 2 hours	0.03	100 - 150	186	1.49
Sequencing-by-synthesis	PCR	ABI 3730 capillary	650	2 hours	9.6 x 10-5	0.06	89	1490.45

NGS technologies can be categorised into 2nd and 3rd-generation platforms. Second-generation are currently more widely used, including Roche 454 pyrosequencing and Illumina sequencing-by-synthesis and require the amplification by PCR of templates before sequencing. Third-generation technologies, including those supplied by Pacific Biosciences, directly sequence single molecules of DNA without prior amplification. The process of NGS typically occurs in three steps: DNA library preparation, DNA library amplification and sequencing.

6.1.3 Library preparation for NGS

Many methods are available for preparation of DNA libraries for sequencing and this is usually platform and application-dependent. Most sequencers require the addition of 'barcode' oligonucleotide sequences to the target DNA regions in order to identify each sequence from a pool after sequencing. Adapter sequences are also added to target DNA in order for it to bind to the substrate used for library amplification; typically a slide or bead. For whole genome sequencing, genomic DNA is fragmented and barcode and adapter sequences are added to the fragments.

In the case of 16S rRNA amplicon sequencing, libraries can be prepared in 2 ways. The first method involves using custom primers; the region of interest is amplified with primers that have custom-designed barcode and adapter sequences attached. The Illumina method of library preparation using custom primers is shown in Figure 6.1. Primers are designed that consist of a standard primer sequence for a region of

the 16S rRNA gene linked to a barcode sequence for identification of individual amplicons and to an adapter sequence complimentary to oligonucleotide fragments on a flow cell for the Illumina instrument (145), (334). These long primers are amplified with the target DNA and all amplicons are pooled to make a library. The advantages of this method include the capacity to sequence as many amplicons as barcodes can be designed, potentially allowing many hundreds of samples to be sequenced at once. However, barcode-adapter linked primers are currently relatively expensive (approximately £40 each), so this may be a limiting factor.

In the alternative method, target DNA can be amplified using kit-provided primers, with barcodes and adapters ligated afterwards. The disadvantage of this method is that ligation of adapters often causes 50 bp of both ends of the amplicon to not be sequenced, as the adapters do not ligate exactly on the end of the fragments. This can lead to less discriminatory power when identifying bacteria.

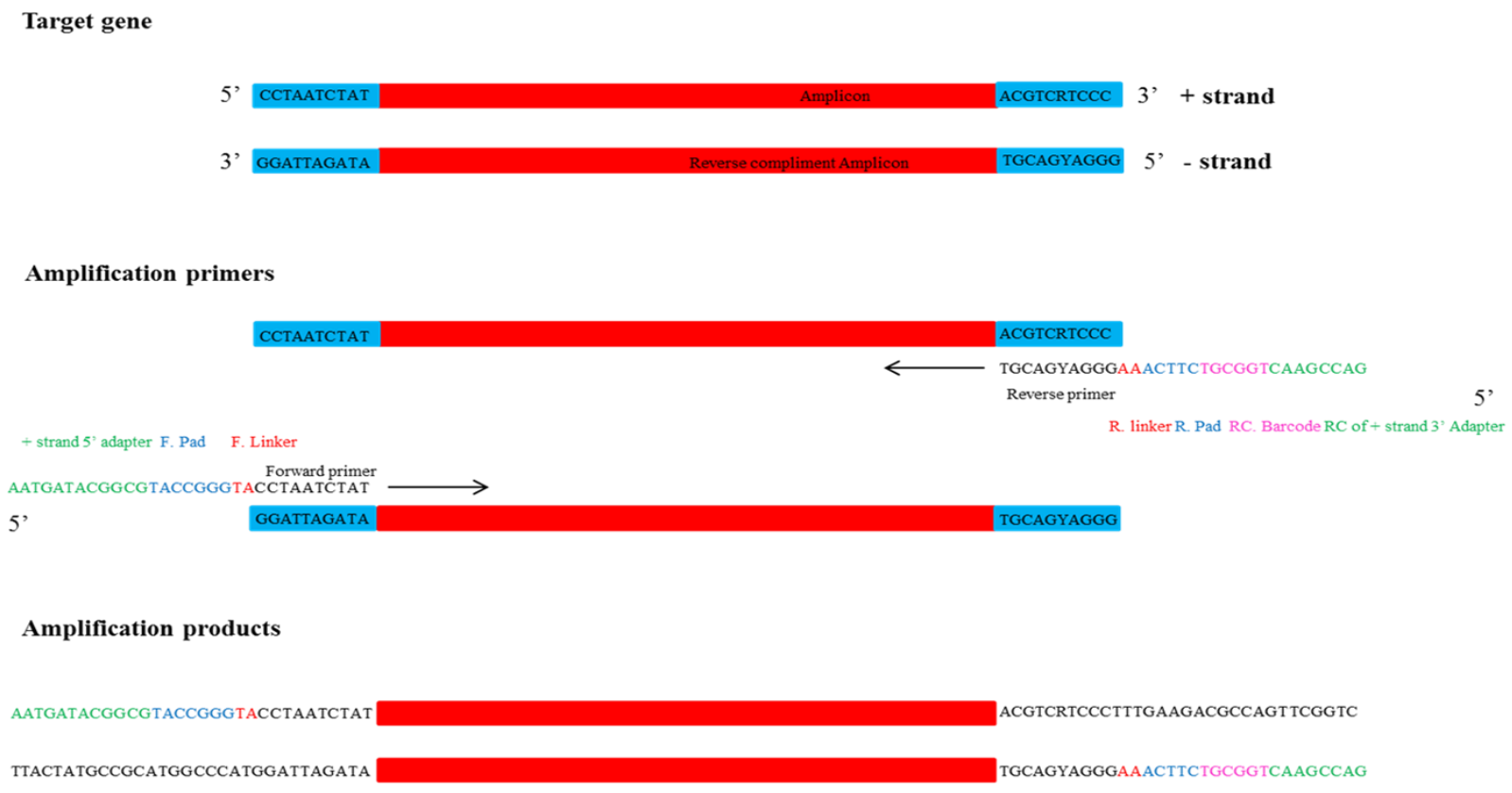


Figure 6.1: Diagram of Illumina barcode primers. Primers complimentary to the region of interest have barcodes, linkers and adapters attached.

6.1.4 Library amplification for NGS

For most NGS platforms, sequencing involves the detection of a by-product created or cleaved when DNA strands are synthesised. Most detection systems within NGS platforms cannot currently identify single nucleotide incorporation or termination events and therefore target DNA regions are amplified prior to sequencing. The two most common amplification methods are emulsion PCR (emPCR) and solid-phase amplification, also known as 'bridge PCR'. This pre-amplification is not required for 3rd-generation platforms such as the PacBio RS as it can sequence from single molecules.

6.1.4.1 Emulsion PCR

The 454, Ion torrent and SOLiD systems use emPCR to amplify DNA libraries prior to sequencing. After library preparation, single strands of template DNA from the library are bound to beads by adapter-complimentary strands on the surface. PCR reagents and single beads are encapsulated in water-oil emulsion droplets and each droplet serves as a 'micro-reactor' in which clonal PCR occurs (335). It is thought that by separately containing each individual PCR reaction, the generation of chimeric sequences is reduced compared to other PCR methods (336), (337).

6.1.4.2 Bridge amplification

Illumina are currently the only company to use bridge PCR to amplify DNA libraries. A flow cell has bound oligonucleotide sequences which are complimentary to the adapter sequences present on template DNA after library preparation. DNA amplification occurs and the resulting dsDNA loops over to form a bridge by binding to another bound oligonucleotide. The dsDNA molecules are denatured and the process starts again. Each amplified strand is clonal and bound near to others in clusters, this can potentially occur at millions of sites (tiles) on the flow cell. After the last denaturation step, free DNA ends are available for sequencing primers to bind (Figure 6.2).

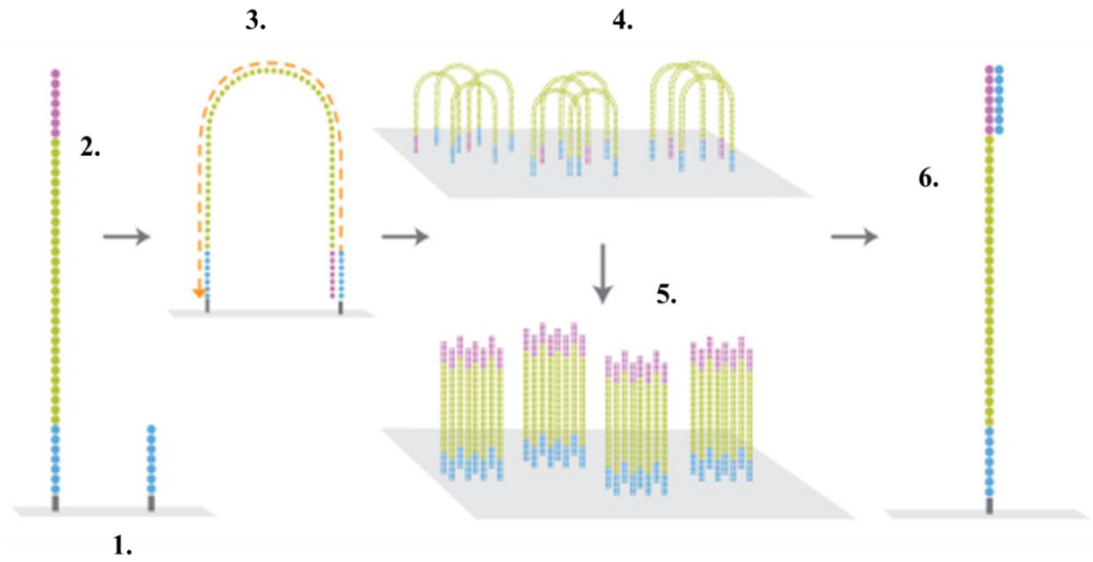


Figure 6.2: Bridge amplification of DNA. 1) Oligonucleotides are bound to the flow cell. 2) Template DNA attaches. 3) Amplification occurs and strands loop over to form bridges. 4) Clusters of bridged DNA form on the flow cell. 5) DNA is denatured. 6) Sequencing primers attach. Image from www.illumina.com

6.1.5 Sequencing approaches for NGS

Once DNA libraries have been prepared, they are applied to a platform for sequencing. Different strategies, as outlined in Table 6.1 are available for sequencing.

6.1.5.1 Pyrosequencing

Pyrosequencing, as carried out by the 454 platforms, measures the release of pyrophosphate molecules as new DNA strands are synthesised (338). Beads from the emPCR are applied to a picotitre plate (one bead per well) and 4 deoxyribonucleotide triphosphates (dNTPs); dATP, dGTP, dCTP and dTTP are washed over the plate sequentially. If a dNTP is incorporated, it will release pyrophosphate and in a series of enzymatic reactions, results in the generation of light. As the target DNA has been amplified previously by emPCR, the light is given off from millions of the same strand providing levels that can be detected by an imager.

6.1.5.2 Sequencing-by-synthesis: cyclic reversible terminator chemistry

After DNA is amplified by bridge-PCR, clonal clusters of amplicons are denatured to form single strands. Sequencing primers, DNA polymerase and modified dNTPs are applied to the flow cell. dNTPs are bound to a dye and are modified to terminate DNA chain synthesis. After incorporation of a nucleotide, the flow cell is imaged to

detect the dye. The dye and terminating group are then removed, leaving a normal nucleotide which becomes incorporated into the growing strand, allowing the process to be repeated (339), (340).

6.1.5.3 Sequencing-by-synthesis: ion semi-conductor technology

Ion Torrent platforms use a unique method to detect nucleotide incorporation when sequencing-by-synthesis. Libraries are prepared by emPCR and beads are applied to wells on a conductive silicon chip. Nucleotides are washed over the chip and if incorporated, the naturally occurring chemical reaction to bind the nucleotide to the growing strand releases a hydrogen ion (H^+). The proton alters the pH of the fluid surrounding the bead and detectors under the well register this and use it to call the base (341). This method does not rely on the use of expensive imaging hardware, making the cost of the machine cheaper than other platforms.

6.1.5.4 Sequencing-by-ligation

Sequencing-by-ligation is the strategy used by the SOLiD platforms. After emPCR, sequencing primers hybridise to adapters on the DNA template. Four fluorescently labelled probes compete to bind to the sequencing primers. They are attached by DNA ligase. After each sequential addition of the labelled probes, imaging is carried out to call the base and then the probes are cleaved, washed away and the cycle is repeated (342).

6.1.5.5 Single-molecule templates

The sequencing of single molecules of nucleic acid avoids biases introduced by PCR methods. DNA polymerase is hybridised to a solid support and produces a complimentary strand to the template, giving off fluorescence as each nucleotide is added (343). This method has only very recently been shown to be applicable to analysis of microbial communities due to reductions in error rates previously reported (344).

6.1.6 NGS platform choice

Very little independent verification and comparison work has been carried out on available NGS technologies, especially in the case of 16S rRNA microbial community analysis. This is largely due to the ever-changing chemistries, software and the cost of purchasing platforms and reagents (333). Even when studies are conducted, the results rapidly become redundant as new technologies emerge (329,345). Platforms give different data outputs and have different ways of assessing quality of sequencing. This means that a variety of bioinformatics tools are needed to analyse data obtained, making comparative work difficult (328).

At the time of sample processing for this project, the Illumina MiSeq v.2 kit had just been launched which provided the longest read length (2 x 250 bp) and depth of sequencing at a relatively cheap price in comparison to other platforms. No work had been published using the Illumina MiSeq to investigate indoor environmental

bacteria and this is currently still the case with regard to hospitals. As such there was no direct reference material for protocols and experimental design. However, the Caporaso et al. protocols and the continued research by his and other groups facilitated the use of this new technology. NGS users have also developed a number of on-line communities in the form of blogs, news feeds and forums, which allows the rapid sharing of knowledge and information regarding these cutting edge techniques.

6.1.7 Limitations of NGS for community analysis

Despite the large increase in published work using NGS technologies in recent years, the techniques have a number of disadvantages. Microbial community studies still largely rely on PCR and this can introduce biases or lead to chimera generation and influence identification (216), (346), (347). Accurate identification of bacterial species is difficult with lower-cost, shorter read platforms and genus-level identification is most commonly reported. Also, error rates for NGS platforms are often higher than for Sanger sequencing (348). The rapid development and wide-range of available technologies means that data obtained is very difficult to compare across the literature. For community studies, different regions of the 16S rRNA gene are often used with different library preparation and sequencing methods, meaning that comparisons are impossible. One of the main limitations to NGS lies in the data analysis. Many different pipelines are available, making research hard to compare but also the lack of skilled bioinformaticians in many laboratories means there is a risk of spurious information being added to public databases (349). The cost of data

analysis can also be prohibitive and must be taken into consideration when planning NGS studies.

6.2 Chapter aims

Understanding bioburden and spatial distribution of microorganisms within a hospital ward may allow the implementation of targeted intervention strategies to reduce environment-mediated transmission of pathogens. Part of the work presented in this chapter is the assessment of spatial and temporal variability of bacteria. TVCs taken over a year aim to determine if 'problem areas' are identifiable or if physical or temporal parameters have an influence on bioburden. Long-term data will also determine whether TVC counts are a useful method of assessing contamination on a ward and whether they can be used to assess risk to patients. The second main aim of this chapter is to understand more about the composition of microbial communities present within a hospital ward, using NGS and to determine if spatial and temporal differences occur.

6.3 Methods

6.3.1 Site selection

Sampling of a high-dependency neurology ward (Koala ward) at GOSH was carried out prior to the ward opening, 2 weeks after opening and continued for a further 12 months.

Samples were taken once a month on either a Monday or Tuesday at 09.00. Enrichment swabs, as used in Chapter 4 were no longer included. Sample size on Koala was increased to 16 swabs and 16 contact plates per bed space (total 64 per time point) due to the increased size of the ward compared to Tiger ward in Chapter 4. Sample site choice was based upon the sites being fixed so as to be directly comparable between time points. A range of heights and materials were sampled across the ward as well as high and low touch frequencies. Table 6.2 lists the sample sites for Koala ward and they are shown in Figure 6.3 with Figure 6.4 showing a bed space in more detail.

Table 6.2: Sampling sites on Koala ward

Sample number	Sample site	Sample number	Sample site
1	Chair arm	**9**	Bin
2	Over-bed light	**10**	Top of alcohol / soap dispenser
3	Drawers, middle level	**11**	Sink
4	Entertainment monitor	**12**	Patient table
5	Middle window ledge	**13**	Bed rail
6	Bottom window ledge	**14**	Patient extendable light
7	Floor under bed	**15**	Drug cabinet
8	Drawers, bottom level	**16**	A & B: high window ledge, C& D: Trolley

The ward was divided into 4 areas (A - D) to facilitate sample collection and each sample was given a unique identifying code as follows:

- **Prefix:** C (contact plate), S (swab)
- **Location code**: W (ward)
- **Ward code**: K (Koala)
- **Month Code:** Jan - Dec
- **Area of room:** A - D
- **Sample number:** 1-16

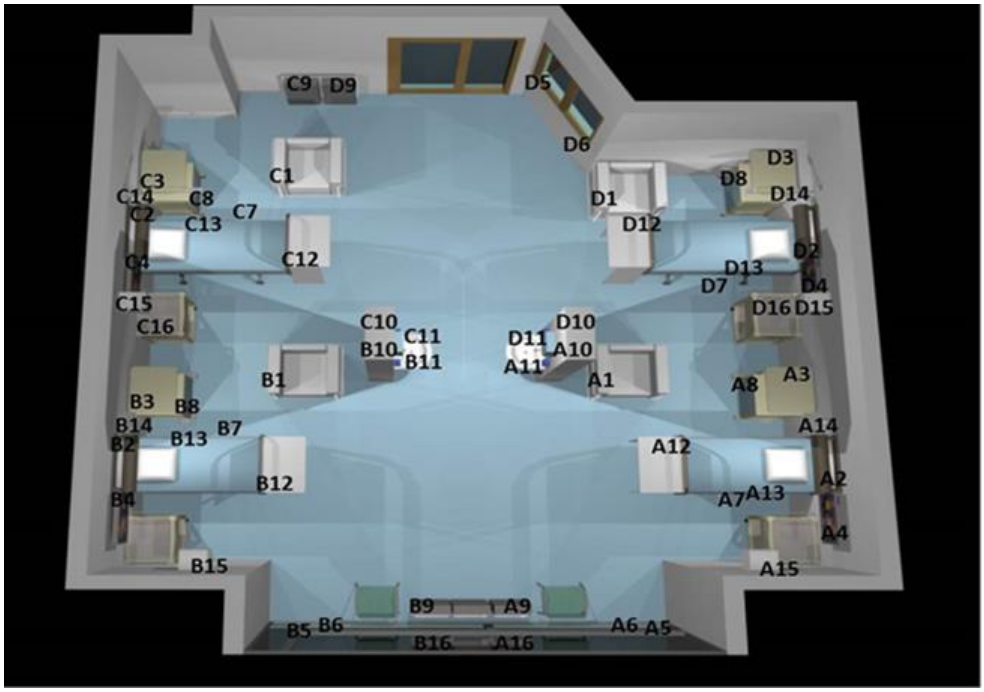


Figure 6.3: Sampling sites on Koala ward

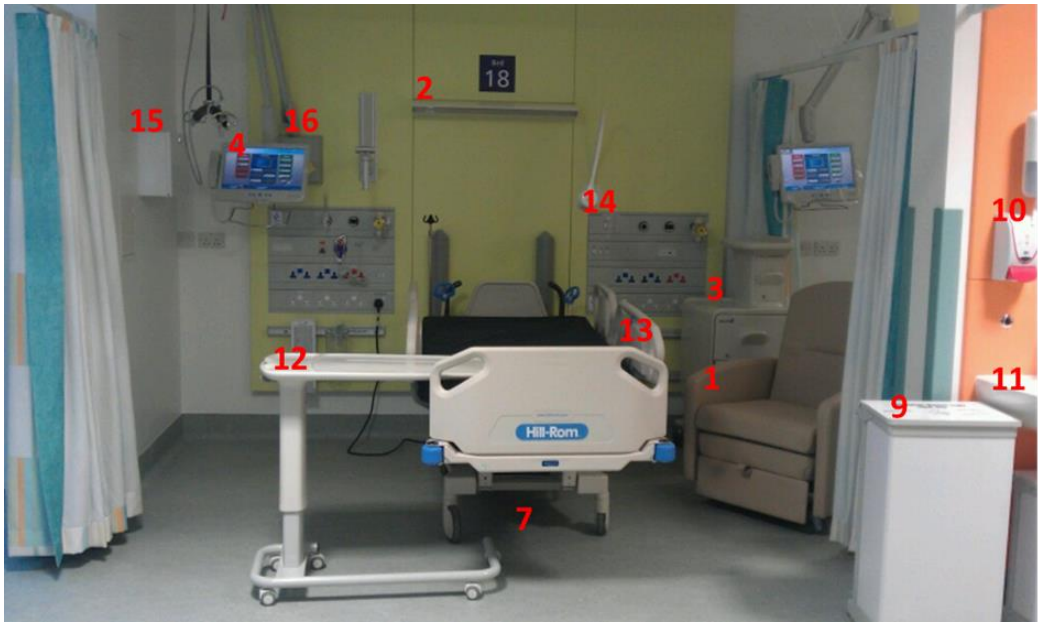


Figure 6.4: Koala ward bed space showing sampling sites

Information regarding whether the bed was occupied or unoccupied was recorded, as was whether the patient had an active bacterial infection and / or an infection control microbial alert.

6.3.2 Environmental sampling

TSA contact plates, swabs, air samples and temperature and RH readings were taken and processed as detailed in Section 2.2.

6.3.2.1 Cleaning

Cleaning was carried out in accordance to DH guidelines and local policies. Standard cleaning took place between 07:30 and 15:30 each day. Floors were dry-mopped with a dust-control mop, bed rails, fixtures and fittings were wiped with a damp cloth containing detergent. At mid-day, cleaners were required to wipe sinks and damp-wipe other surfaces if necessary. Between 16:00 and 20:00, sinks were damp-wiped again and the floor was dry-mopped. Throughout the day, nursing staff were required to wipe bed space areas with 'Tuffie' bactericidal wipes. Weekly cleaning involved damp-wiping with detergent under bed frames and high surfaces, including walls. Cleaning was carried out to comply with visual inspection standards of being free of dust, debris and finger marks. Bed spaces were cleaned to a level 3 standard on discharge of a patient with a microbial alert. This involves the use of 1000 ppm chlorine and is also referred to as a 'deep clean'.

6.3.3 Identification of *Pseudomonas aeruginosa* from sinks

Frozen colonies from TVC plates taken on the sinks were thawed onto MacConkey agar (Oxoid, UK) and incubated at 37 ^{O}C for 24 hours. Colonies were picked and streaked out again on MacConkey agar to obtain pure cultures. Those colonies with morphologies indicative of *Pseudomonas* growth (i.e. white / colourless / yellow and circular) were sub-cultured on to cetrimide agar (Oxoid, UK) and incubated at 42 ^{O}C overnight to select for *P. aeruginosa*.

Colonies were viewed under UV-light and those fluorescing were sub-cultured onto 2 plates of ISO Sensitest agar and one plate of UTI-chromogenic agar (both Oxoid, UK). ISO Sensitest agar plates then had drug-impregnated discs added to them (Table 6.3) and were incubated at 37 ^{O}C overnight.

Table 6.3: Antibiotics used to test for *Pseudomonas aeruginosa* resistance

	Antibiotic	Concentration (μg)
Plate 1	Gentamicin	10
	Ciprofloxacin	5
	Ceftazidime	30
	Piperacillin / Tazobactam 75:1	85
	Meropenem	10
Plate 2	Imipenem	10
	Tobramycin	10
	Aztreonam	30
	Colistin	25

6.3.4 Sequencing of colonies from TVC plates

In order to identify the most commonly observed bacterial colonies growing on TVC plates, colonies were picked and sequenced. DNA was extracted using the method given in Section 2.5.1. 16S rRNA PCR was performed as described in Table 2.2 and PCR clean-up carried out using Sephadex™ columns (Section 2.8.2). Sequencing was carried out as described (Section 2.10.1) and analysed using BLAST (Section 2.10.3.1).

6.3.5 PCR inhibition assay

At the time of DNA extraction from swabs, mouse DNA was added as an extraction control (Section 2.5.2). A qPCR assay was carried out on each sample before pooling to check for the presence of inhibitors by amplification of the mouse DNA (Table 2.8). Any samples with a C_t of > 30 were considered to be inhibited. These samples were diluted at 1:10 and the assay was run again. If the C_t was still > 30, the sample was not included in the pool, if it was < 30; the 1:10 dilution was added to the pool. Details of sample pooling are given in Section 2.10.2.1.

6.3.6. MiSeq DNA sequencing

A total of 2 MiSeq sequencing runs were carried out for the current project. The initial run was carried out using the protocol given in Section 2.10.2.2 and the second using the protocol for low concentration DNA libraries as given in Section 2.10.2.7. A total of 96 samples were combined at a concentration of 2 nM for the initial run. For the second run 50 samples were used. 32 of these amplified well and were purified using the Size Select® gel method. The remaining 19 did not amplify well and PCR products were pooled and concentrated on a QIAquick column. The samples that amplified well were each adjusted to 1 nM concentrations and combined. The concentrated pool of poorly amplifying samples was added to the library at the same concentration. Pooled libraries were checked for final concentration and purity on a Bioanalyzer, using the method given in Section 2.9.3.

6.3.7 Statistical analysis and bioinformatics

Statistical analysis was performed using either IBM SPSS Version 21 or R-3.0.2. Bioinformatics was carried out as detailed in Section 2.10.3.2.

6.4 Results

6.4.1 Bed occupancy and microbial alerts.

Table 6.4 shows the bed occupancy and microbial alert status of patients over the year-long sampling protocol. Patients with active bacterial infections or colonisations were recorded.

Patients had a wide variety of bacterial infections during the sampling time period, including infection with pathogens commonly associated with HCAIs such as *P. aeruginosa*, *S. aureus*, *K. pneumoniae* and drug-resistant *E. coli* (Table 6.5). These were diagnosed by the Microbiology department at GOSH using standard culture and PCR methods.

Table 6.4: Bed occupancy and infection status on Koala ward during year-long sampling.

	Bed space			
Time Point	A	B	C	D
K1 Baseline	No patient	No patient	No patient	No patient
K2 March	Occupied	Occupied	Occupied	Occupied
K3 April	No patient	No patient	Occupied	No patient
K4 May	No patient	No patient	No patient	No patient
K5 June	Occupied	No patient	Occupied	No patient
K6 July	Occupied	Occupied	Occupied	Occupied
K7 August	Occupied	No patient	Occupied	No patient
K8 September	No patient	No patient	Occupied	Occupied
K9 October	Occupied	Occupied	Occupied	Occupied
K10 November	Occupied	Occupied	Occupied	Occupied
K11 December	Occupied	Occupied	Occupied	Occupied
K12 January	Occupied	Occupied	No patient	No patient
K13 February	No patient	No patient	No patient	No patient
K14 March	Occupied	Occupied	Occupied	Occupied

Blue; Patient had active bacterial infection or colonisation at time of sampling. Red; patient had active bacterial infection or colonisation and official infection control alert.

Table 6.5: Microorganisms isolated from infected or colonised patients during year-long screening of Koala ward. Bed spaces with patients with infection control alerts indicated in red.

Time point	Bed space	Positive culture	Organism
K5	A	Tip culture	Resistant CNS[1]
	C	Wound swab	Resistant CNS
K6	A	Throat swab	Coliforms
	C	Umbilical swab	*Enterococcus*, *S. maltophilia*
		Blood	*K. ozaenae*, *K. pneumoniae*
K7	C	ETA[2]	*Burkholderia cepacia* complex
		Throat swab	*B. cepacia*
		Sputum	*Serratia* spp., coliform, *Candida*
		Urine	Unidentified growth
		Throat swab	*Serratia* spp.
K8	C	Wound swab	CNS
		Pus	*P. aeruginosa*, coliform
		Pus	*P. aeruginosa*, coliform, *Prevotella*
		Wound swab	*S. marcesens*, *P. aeruginosa*
		Scalp swab	Coliform, *P. aeruginosa*
	D	Nasal swab	*S. aureus*
		CSF[3]	β-haemolytic *Streptococcus*, Group A
		Urine	*Candida*
		Faeces	*E.coli*
K9	D	Femoral line	Coliform
		Faeces	*E.coli*, other coliform
		Faeces	Sapovirus
		Subdural fluid	Group A *Streptococcus*
		Urine	Mixed, *Candida*
		NPA[4]	*B. cepacia* complex, Gram negative rod
K10	B	Throat swab	Coliform
K11	A	Dermatoid cyst	*Kocuria kristinae*
	B	Faeces	*E.coli*

1) Coagulase-negative staphylococci. 2) Endotracheal aspirate. 3) Cerebrospinal fluid. 4) Nasopharyngeal aspirate.

6.4.2 Total viable count results

Despite attempts to collect a full set of 64 TVC plates per time point from Koala ward, a range of between 34 and 62 plates were collected. A total of 701 plates were taken over the course of the year. An additional CFU count category of 'zero CFU' was added after the investigation presented in Chapter 4, due to there being a larger overall number of samples and a large number of plates having zero CFU.

The percentage of sites with TVCs in each of 5 categories for 14 time points is shown in Figure 6.5. Overall, CFUs recovered tended to be in the zero or < 50 categories. At K1, the baseline sampling, 7 % of plates had zero CFUs and 85 % had between 1 and 49 CFUs. This changed at the first time point after ward opening (K2) to 40 % of sites having between zero and 49 CFU, with the majority of the remaining sites having intermediate to high CFUs. By K3, the majority of sites again had returned to having low numbers of CFUs. This changed again over the next 2 time points but remained around 30 – 50 % for subsequent time points until declining again at K12 and K13 and increasing slightly at the final time point.

Number of sites with > 200 CFUs recovered remained fairly constant with a small peak at time points K5 and K6, but they remained under 10 % of the total for the rest of the time points. This was also true of the numbers of sites with 101 – 200 CFUs, overall the percentage of sites was higher than for the > 200 category but after K6, the percentage of sites having this number of bacteria remained around or under 10

%. There was an increase at K2, and a larger peak to 26 % at K5. Number of sites having 50 –100 CFUs appeared inversely proportional to number of sites with 1–49 CFUs. If the former was higher, the latter lower and vice versa.

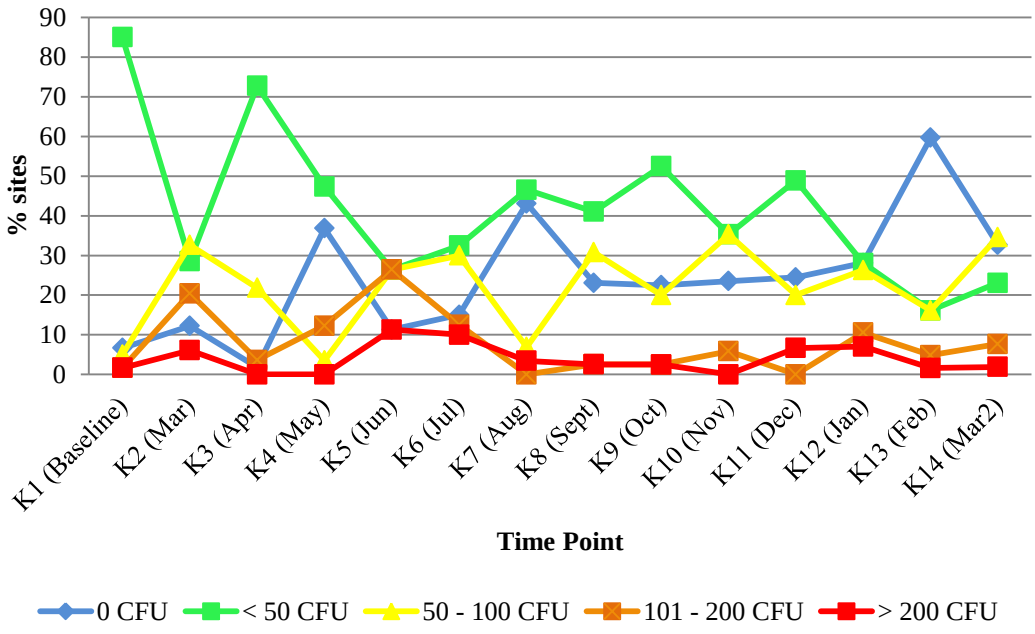


Figure 6.5: Percentage of sampling sites with bacterial colony-forming units in each of 5 categories over a year on Koala ward.

At time point K13, 60 % of sites sampled had zero CFUs, giving it on average, the same TVC count as the baseline sampling point, prior to occupancy. K3 had the least number of sites with zero CFUs. From the first sampling time point after ward opening in March 2012 (K2) to the final sampling exactly a year later in March 2013 (K14), the percentages of sites having CFUs in each category was similar, except for an increase in the percentage of sites having zero CFUs from 12 to 33 % and a decrease in sites with > 200 CFU from 6 to 2 %.

67 % of all TVC contact plates taken from the ward over the year had low or no CFUs present (472 of 701 plates).

When analysing total average TVCs from all sites sampled across time points, it can be seen that after the baseline screen, the total number of CFUs on the ward increased dramatically (Figure 6.6). However, at the next time point (K3), overall numbers declined again and returned to approximately baseline level at K4. There was a large increase in overall TVCs at K5 and K6 but this returned to baseline again at K7. The CFU numbers then began to rise slowly before dropping again at K13 to slightly higher than baseline numbers.

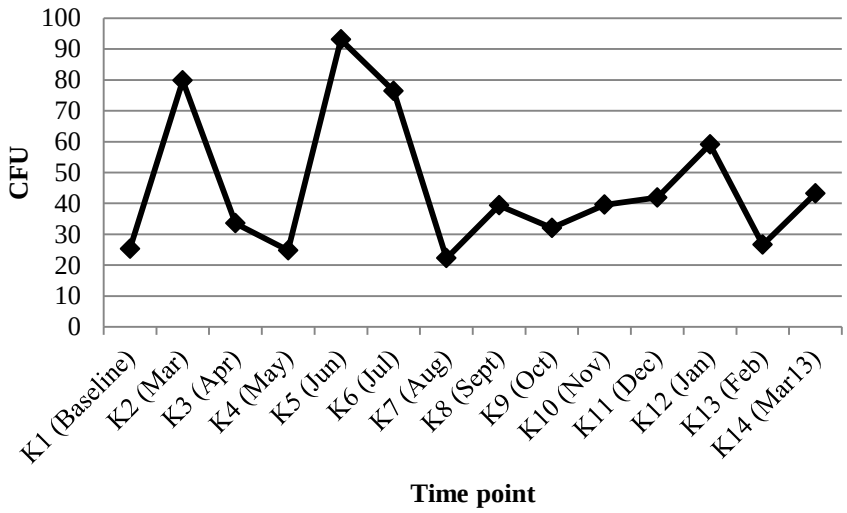


Figure 6.6: Average total viable counts of bacterial colony-forming units on Koala ward over a year.

In order to analyse any patterns that may occur with CFU recovery in relation to sample type over the course of the year, the sample set was arranged into sites

immediately near to the bed space, non-bed space sites, floors and sinks. Average CFUs per bed space were plotted to identify if bed space was correlated to CFU recovery (Figure 6.7).

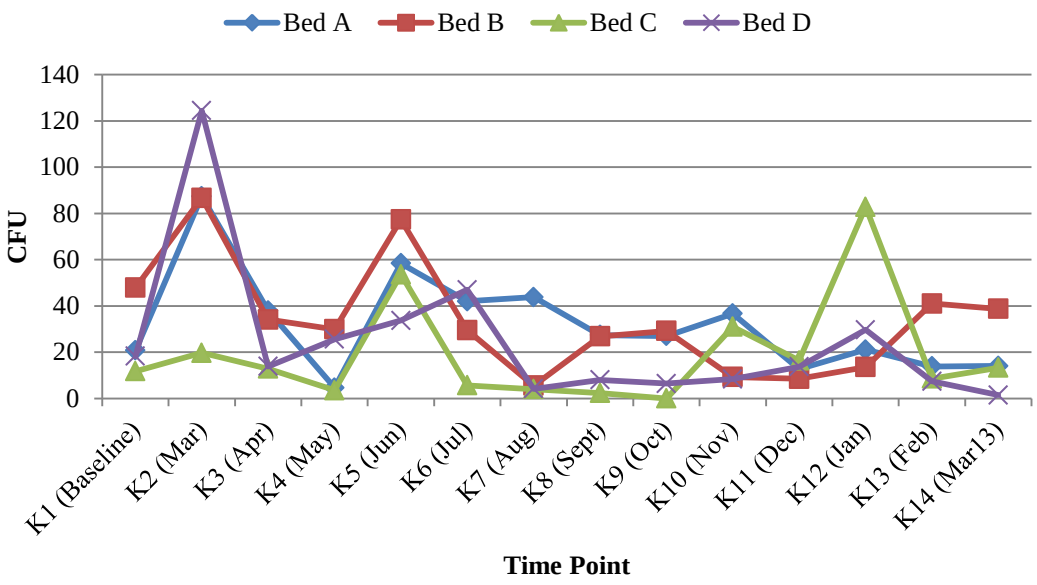


Figure 6.7: Bacterial counts recovered from each bed space on Koala ward over a year.

Bed space B had slightly higher CFU counts than other bed spaces at the baseline time point and all bed spaces except C had the same large increase at K2 compared to K1 (Figure 6.7). CFUs then generally reduced at K3 and K4 time points but increased at K5 for all bed spaces. They were reduced again at K6, which differs from the pattern of total ward CFU averages (Figure 6.6). The small increase in total ward CFUs at K12 (Figure 6.6) can be observed in bed space C but the increase is not as large for the other bed spaces.

Figure 6.8 shows the average bacterial counts from bed space, non-bed space sites, floors and sinks for the year. Average TVCs for non-bed space areas had the same pattern as total TVC counts for the whole ward (Figure 6.8b). Bed spaces, sinks and floors did not show the same pattern. Floor TVCs remained low for the first 2 months after baseline screening but then increased at K4 and peaked even higher at K5 (Figure 6.8c).

This was followed by a steady decline to K9, after which CFU numbers increased and decreased each month. CFUs recovered from sinks showed no pattern and increased and decreased almost monthly but peaked in December at K11 (Figure 6.8d).

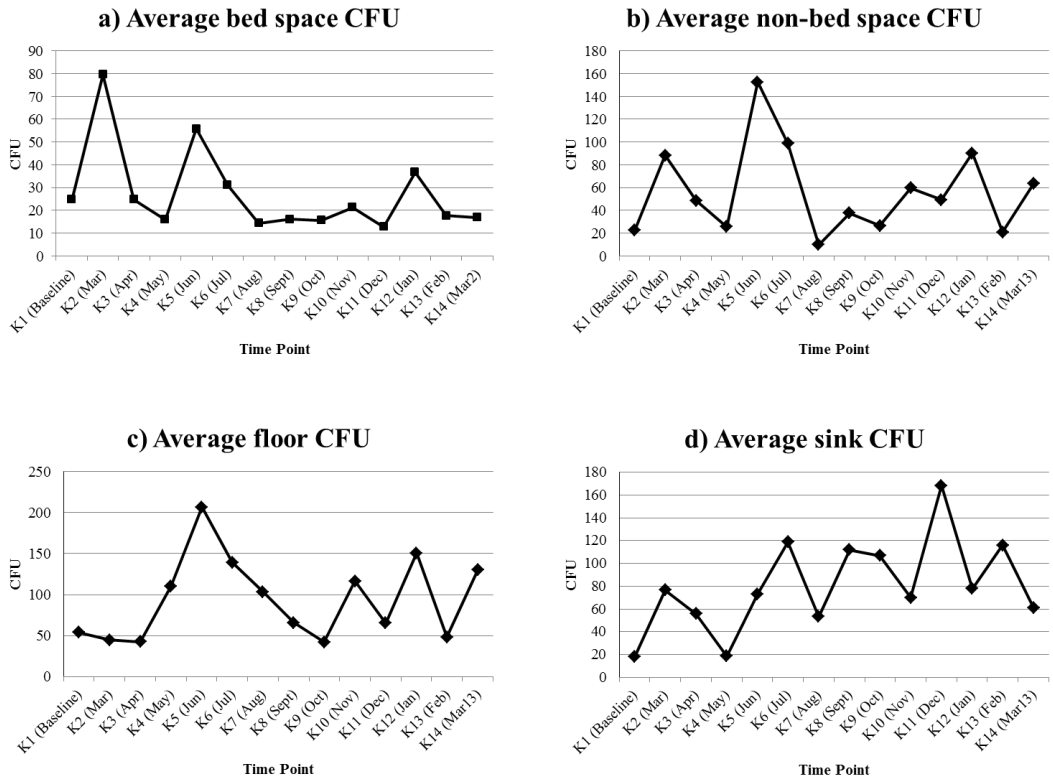


Figure 6.8: Average total viable counts recovered from a) bed spaces, b) non-bed space sites, c) floors and d) sinks over a year.

With relation to bed occupancy, average TVC counts showed no difference between occupied and unoccupied beds (p = > 0.05). Occupied bed spaces did not have higher TVCs than non-occupied bed spaces. However, when a patient with a microbial alert was present, TVC counts from their bed space were always significantly lower than other bed spaces on the ward (Table 6.6) (p = < 0.05).

Table 6.6: Bed space occupancy and average total viable counts recovered from Koala ward at all time points.

Time point	Bed space	Occupancy	Ave. CFU	Time point	Bed space	Occupancy	Ave. CFU
K1 Baseline	A	No patient	21	K8 September	A	No patient	27
	B	No patient	48		B	No patient	27
	C	No patient	12		C	Occupied	2
	D	No patient	18		D	Occupied	8
K2 March	A	Occupied	87	K9 October	A	Occupied	27
	B	Occupied	87		B	Occupied	29
	C	Occupied	20		C	Occupied	0
	D	Occupied	124		D	Occupied	6
K3 April	A	No patient	38	K10 November	A	Occupied	37
	B	No patient	34		B	Occupied	9
	C	Occupied	13		C	Occupied	31
	D	No patient	14		D	Occupied	8
K4 May	A	No patient	5	K11 December	A	Occupied	13
	B	No patient	30		B	Occupied	9
	C	No patient	4		C	Occupied	16
	D	No patient	26		D	Occupied	14
K5 June	A	Occupied	58	K12 January	A	Occupied	21
	B	No patient	77		B	Occupied	14
	C	Occupied	54		C	No patient	83
	D	No patient	34		D	No patient	30
K6 July	A	Occupied	42	K13 February	A	No patient	14
	B	Occupied	30		B	No patient	41
	C	Occupied	6		C	No patient	9
	D	Occupied	47		D	No patient	7
K7 August	A	Occupied	44	K14 March 13	A	Occupied	14
	B	No patient	6		B	Occupied	39
	C	Occupied	4		C	Occupied	13
	D	No patient	4		D	Occupied	1

Blue; Patient had active bacterial infection or colonisation at time of sampling. Red; patient had active bacterial infection or colonisation and official infection control alert

In summary, the majority of sites sampled over the year had TVCs of between 0 - 49 bacteria. Average ward TVCs were low at baseline with a sharp increase at K2 after occupancy and were also high at K5 and K6. This pattern was mirrored in average non-bed space TVCs, but not in bed space, floor or sink averages. No single bed space was consistently high but all except C were high at K2, likely contributing to the high total ward average. All bed spaces had high TVCs at K5 and bed space C was also high at K12. Bed space TVCs were generally lower than the total ward average, except at K2 where they were higher than all other areas. Floors had higher average TVCs than other areas sampled with a peak at K5 and sink TVCs showed no pattern.

6.4.3 CFU counts and temperature and relative humidity

Temperature and RH readings were taken at intervals a minute apart in each of the 4 bed spaces of Koala ward (A – D) at each time point. The average temperature on Koala ward during the sampling period ranged from 21.1 ^{O}C to 25.5 ^{O}C (Figure 6.9). The highest recorded temperature was at K2 in March 2012 and the lowest at K4 in May. RH ranged from 20 % to around 50 % over the summer months (K5 – K7) peaking at 51 % in August (K7). RH declined gradually over the winter months from November (K11) and continued until its lowest value at the final time point. There appeared to be no correlation between temperature and RH in this study (Pearson's correlation coefficient r = -.283, p = > 0.05, df = n-2).

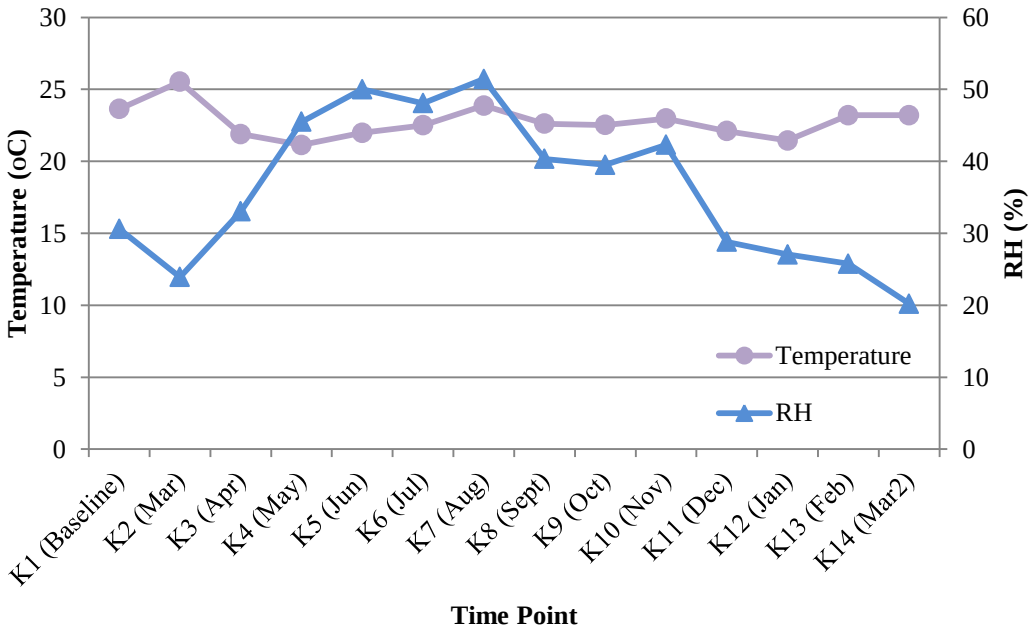


Figure 6.9: Average temperature and relative humidity readings from Koala ward.

6.4.3.1 Total viable count and temperature

Figure 6.10 shows average ward temperature and average ward TVCs (measured as CFU), for the sampling period. Initially, temperature and recovered CFUs followed the same trend, CFUs increased with temperature and the same was true for a decrease. This pattern occurred from K1 until TVCs peaked dramatically in June (K5). At this point, temperature readings also increased but only slightly from the previous month. The temperature continued to rise steadily over the next 2 time points but only by 2 ^{O}C whereas TVC decreased. In September (K8), TVC and temperature began to follow the same pattern again until they became inversely related in January (K12) and February (K13). TVC did not correlate significantly

with temperature (Pearson's correlation coefficient r = 0.084, p = > 0.05, df = n -2) and CFU count could not be predicted by temperature (R^2 = 0.007).

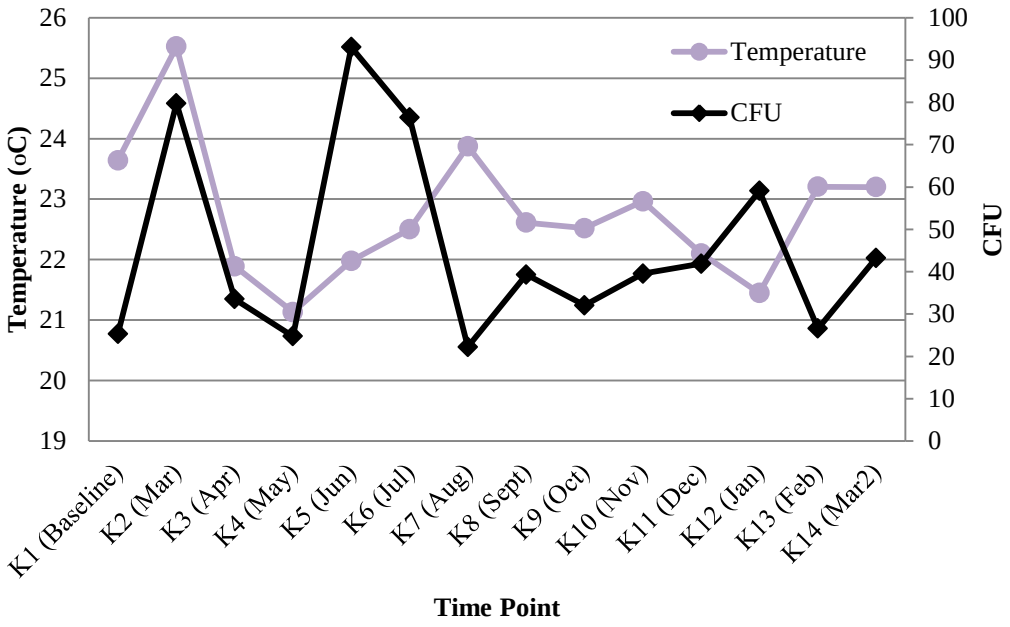


Figure 6.10: Average temperature and average total viable counts on Koala ward.

6.4.3.2 Bed space area, total viable count and temperature

In order to determine if small differences and fluctuations in temperature in local bed space area had an influence on TVC, each bed space was analysed individually. Temperature patterns appeared similar for each bed space, but bed space C had a temperature peak of 26 ^{O}C in August (K7) compared with temperatures of 24 ^{O}C in bed space D and 22 ^{O}C at bed spaces A and B (Figure 6.11). There was a marked decrease in temperature between November (K10) and January (K12) and a rise

again in February (K13) at both bed space A and B (Figure 6.11 a & b). This was also observed in bed space C and D but not as markedly (Figure 6.11 c & d).

Each bed space had the initial trend of TVCs increasing and decreasing with temperature until K5 when CFU numbers increased sharply but temperature only increased slightly. The pronounced temperature peaks at K7 (August) did not correlate with increases in CFUs, in fact CFUs decreased at all bed spaces at this time point. There was no statistical correlation between CFU count and temperature for any bed space.

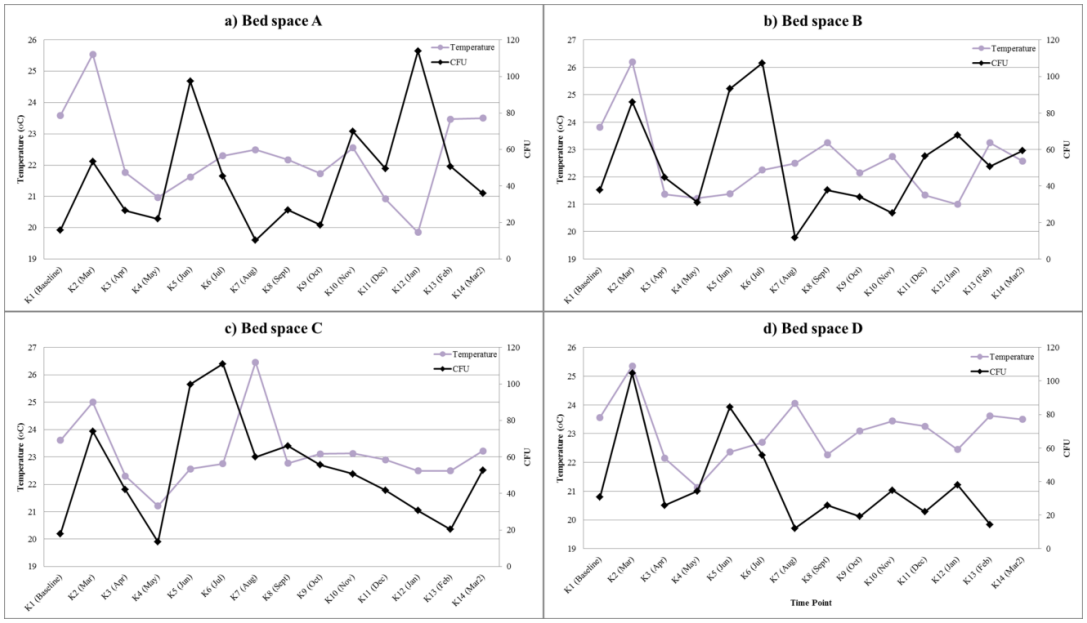


Figure 6.11: Average bed space temperature and total viable count on Koala ward.

6.4.3.3 Total viable count and relative humidity

Figure 6.12 shows average ward RH and average ward TVC (measured as CFU) for the sampling period. RH increased gradually after a small decrease at K2 (March), until an overall decline from K8 (September) to the final sampling point (K14) (Figure 6.12). There was no correlation between average TVC and RH on Koala ward for this sampling period (r = -2.83, p = > 0.05, df = n-2) and CFU count could not be predicted by RH (R^2 = 0.006).

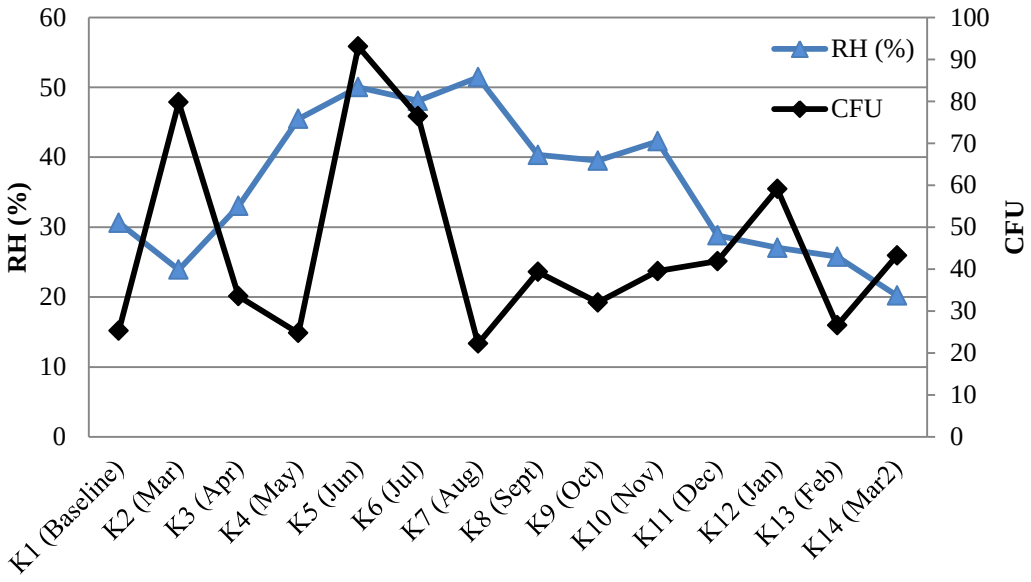


Figure 6.12: Average relative humidity (%) in relation to average bacterial total viable counts on Koala ward.

6.4.3.4 Bed space area, total viable count and relative humidity.

In order to determine if small differences and fluctuations in RH in local bed space area had an influence on TVC, each bed space was analysed separately. RH patterns appeared similar for each bed space, they increased gradually until K7 (August) and then decreased gradually for the remainder of the sampling period (Figure 6.13). TVCs from bed space C appeared to follow a similar trend to RH from K5, with peaks over the summer months until a decline after K8 (September), however, there was no statistical correlation between CFU count and RH for any bed space.

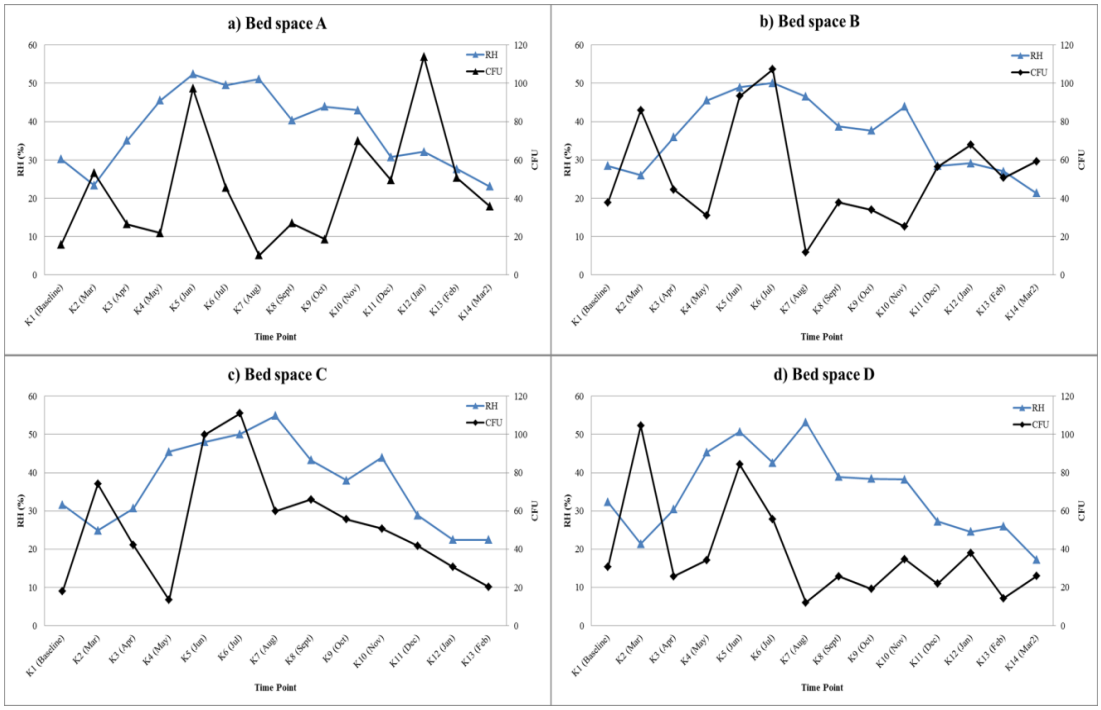


Figure 6.13: Average bacterial total viable counts in relation to relative humidity for each bed space on Koala ward.

6.4.4 Identification of bacteria from TVC plates

In order to understand which bacteria were commonly observed on TVC plates, the most frequently appearing types of colonies were Sanger sequenced. Results are presented in Table 6.7. Figure 6.14 shows the same sample numbers as Table 6.7 and contains images of the sequenced colonies. Colonies resembling *Staphylococcus* were commonly observed (Figure 6.14) and these were identified as coagulase-negative by sequencing. *Pseudomonas* sp. colonies were also common, as were *Bacillus* sp. and *Micrococcus* sp.

Table 6.7: Identities obtained by DNA sequencing of commonly observed bacterial morphotypes on total viable count agar plates.

Sample number	Morphology	Bacteria ID	Max. identity (%)
1	White, flat round, glossy	*Staphylococcus* (CNS)	99
2	Small, yellow, round, smooth	*Micrococcus* sp.	99
3	Yellow / transparent, irregular	*Pseudomonas* sp.	100
4	Large, matt, off-white, flat	*Lysinibacillus* sp.	98
5	Off-white, flat, round, glossy	*Enhydrobacter* sp.	99
6	White, wet, sweet smell	*Pseudomonas* sp.	100
7	White, crackled, dry, raised	*Bacillus* sp.	99
8	Flat, wet, white, irregular	*Bacillus* sp.	99
9	Small, white, round, smooth	*Micrococcus* sp.	99
10	Yellow, translucent, round	*Staphylococcus* (CNS)	99
11	Round, flat, wet, grey	*Bacillus* sp.	99
12	Wet, irregular, off-white	*Bacillus* sp.	99

6.4.5 *Pseudomonas* identification

One sink in April (K3) and both sinks in June (K5), July (K6) and August (K7) were positive for the presence of *P. aeruginosa*. None of the isolates were resistant to any antibiotic tested.

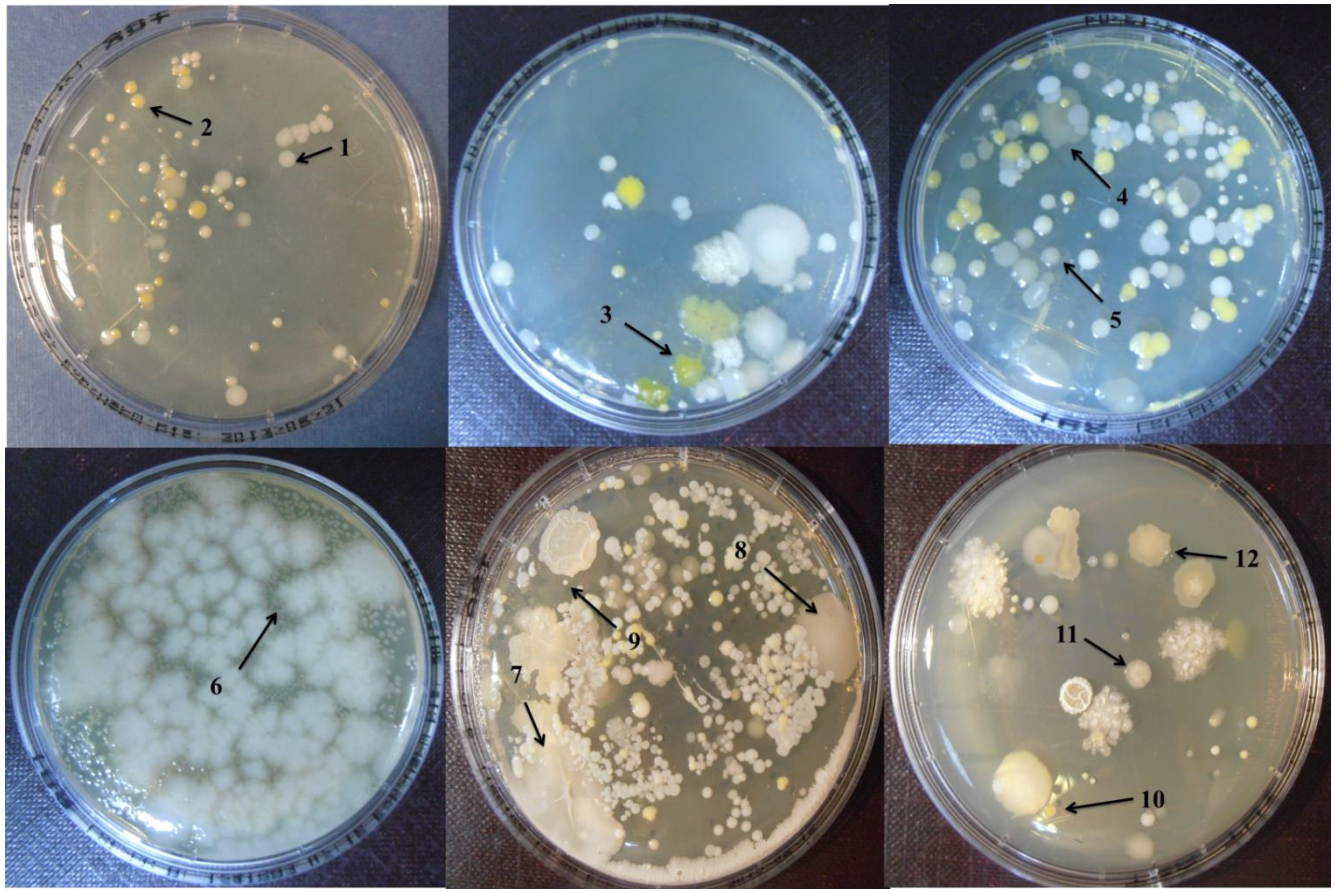


Figure 6.14: Images of total viable count plates from the ward showing colonies sequenced (Numbers refer to Table 6.7).

6.4.6 Next-generation sequencing of samples

The initial sequencing run failed to cluster, meaning no data were retrieved, as discussed in Chapter 3. Fifty samples from Koala ward spanning multiple time points and sample types were included on a second run leading to a total of 629,136 sequence reads passing quality filtering. Of the 50 samples, 24 did not produce an adequate number of reads to be analysed fully (> 1000), leaving 457,777 reads. Of those reads, 3032 were identified as chloroplast DNA and were therefore also excluded from further analysis. No air samples amplified well enough to be included on a MiSeq run.

6.4.6.1 Phylum level identification of bacteria

The 26 samples from Koala ward that were analysed included samples from all areas (bed space, non-bed space, sinks and floors) and multiple time points. They contained sequences which represented 23 known phyla or candidate phyla (those containing uncultured bacteria known only by metagenomics (350)), as seen in Figure 6.15. The highest number of sequence reads belonged the Firmicutes, followed by Proteobacteria and Actinobacteria. Bacteroidetes made up the majority of the remaining phyla with the other 18 phyla being represented by fewer sequences.

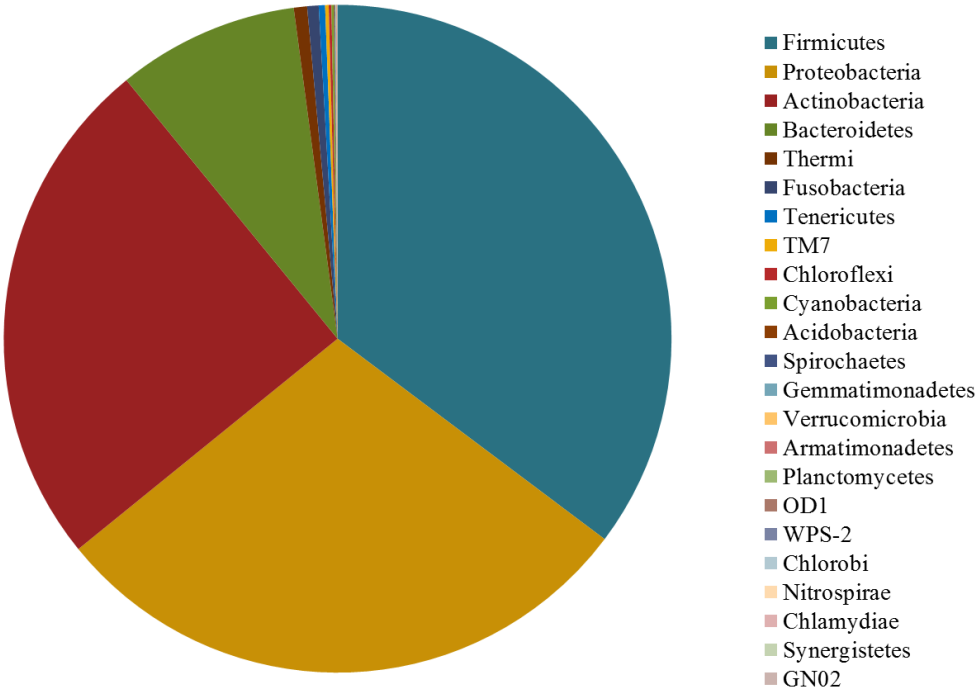


Figure 6.15: Sequencing reads assigned to bacterial phyla isolated from Koala ward.

19 different phyla were identified from bed spaces (Figure 6.16a) with the Actinobacteria comprising 35 % of the reads from the bed space samples. Firmicutes were identified in similar proportions and made up 33 % of the total. Proteobacteria made up 21 % of total bed space sequencing reads and 9 % were assigned to the Bacteroidetes. The remaining phyla, including Tenericutes, Fusobacterium, Thermi and TM7 were found in smaller proportions and others including Acidobacteria, Gemmatimonadetes and OD1 were present in even smaller proportions.

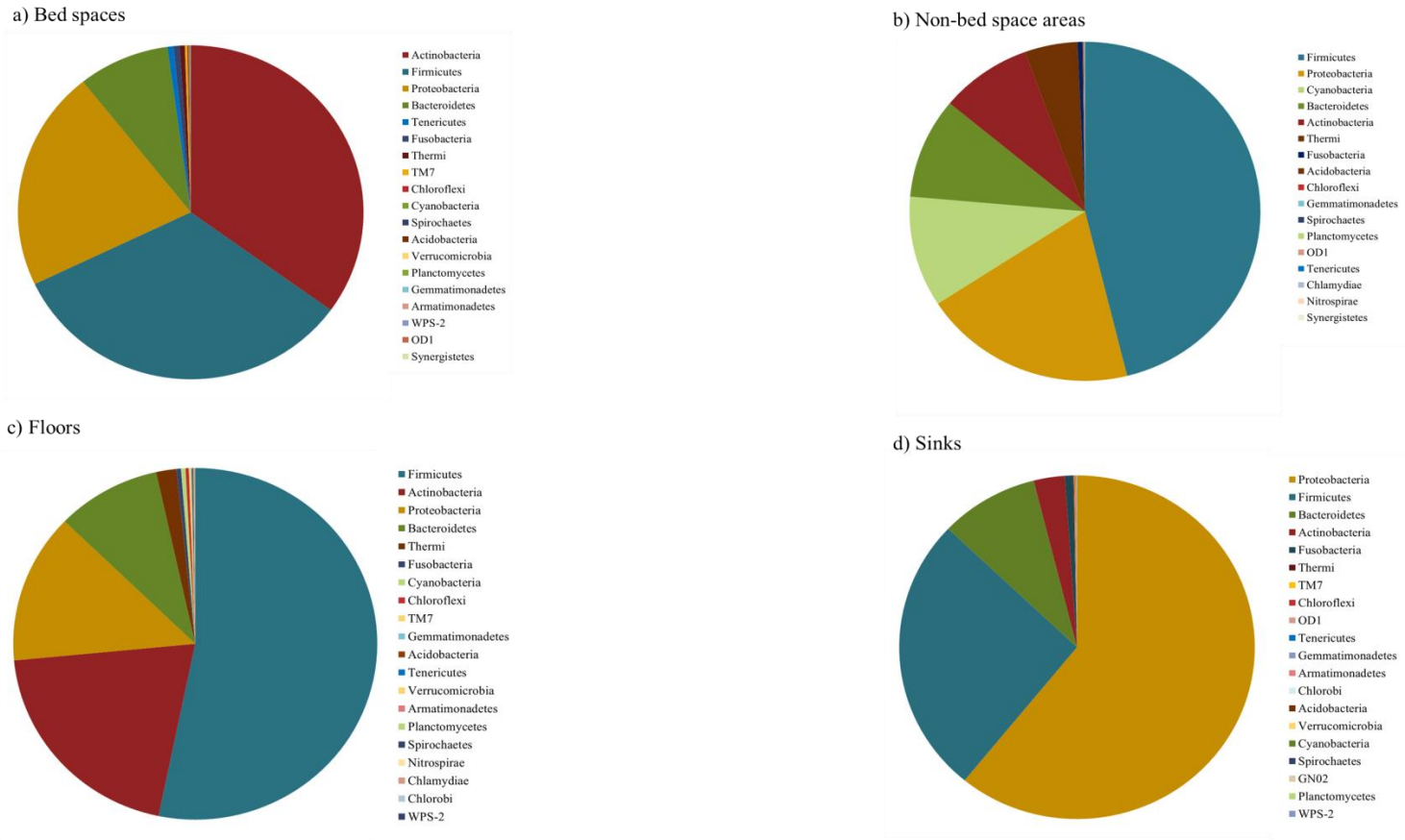


Figure 6.16: Bacterial phyla isolated from bed spaces (a), non-bed space areas (b), Floors (c) and sinks (d) on Koala ward.

17 phyla were identified from non-bed space samples (Figure 6.16b). The Firmicutes made up almost half of the phyla identified at 46 % of sequencing reads. Proteobacteria were present in a similar proportion to bed space samples (20 %) but Cyanobacteria were the next most frequently identified phylum in non-bed space samples at 11 % of reads, in contrast to only being found in 0.07 % of bed space samples. Bacteroidetes and Actinobacter were found in lower proportions in non-bed space areas than bed space areas (10 and 8 %), whilst Thermi and Fusobacteria were found in higher proportions. In contrast to bed space samples, no bacteria from the phyla TM7, Verrucomicrobia, Armatimonadetes or WPS-2 were identified from non-bed space samples but Nitrospirae and Chlamydiae were.

20 phyla were identified from floor samples, with Firmicutes making up over half of all reads (53 %). Actinobacteria, Proteobacteria and Bacteroidetes were present in the next highest proportions, which was similar to the pattern observed in bed space samples (Figure 6.15c). All other phyla present were also either found in bed space and or non-bed space samples.

20 phyla were also identified from sink samples (Figure 6.16d). These samples had a different profile to samples from other areas, with the majority of reads being assigned to the Proteobacteria (57 %). Firmicutes were still present in high proportions, making up 24 % of total sequencing reads. The next most frequent phylum was Bacteroidetes at 8 % of total sequencing reads and Actinobacteria was

found in much lower proportions than in bed space, non-bed space and floor samples, comprising only 2 % of total sequencing reads.

6.4.6.2 Genus level identification of bacteria

Genus-level identification of bacteria present in all samples was determined. Based on current literature and inspection of individual samples, sequences which comprised < 0.05 % of the total reads were discarded, leaving 402,866 reads, representing 231 taxa in the 26 samples analysed. The number of reads observed per sample can be seen in Table 6.8 along with bed occupancy information and the bacterial genus that was dominant, in terms of number of sequences. On average, more taxa were identified in bed space (n=74), non-bed space (n=100) and floor samples (n=94) than sink samples (n=41).

The natural habitats of the identified taxa can be seen in Table 6.9. The majority of taxa isolated from Koala ward over all time points analysed were environmental in nature (122 of 231 identified taxa). These are bacteria normally associated with soil, water, plants or other environmental sources and include taxa such as *Agrococcus*, *Devosia* and *Enhydrobacter*. 63 taxa identified are normally associated with humans, including *Helcococcus* from the skin, *Leptotrichia* from the oral cavity, *Dialister* from the urogenital tract, *Dorea* from the gut and others from other body sites.

Table 6.8: Number of sequencing reads and taxa observed per sample showing the dominant taxon and bed occupancy.

Sample	Reads	Genera	Dominant taxon	Occupancy
K1.E	16012	117	*Staphylococcus*	
K2.A	5981	62	*Streptococcus*	Occupied
K2.E	37265	86	*Staphylococcus*	
K2.F	13675	94	*Staphylococcus*	
K2.S	17635	54	*Streptococcus*	
K3.B	27331	80	*Acinetobacter*	Unoccupied
K3.F	10419	100	*Pseudomonas*	
K3.S	151162	27	*Pseudomonas*	
K4.A	29425	73	*Propionibacterium*	Unoccupied
K4.E	44726	84	*Pseudomonas*	
K4.S	16354	69	*Streptococcus*	
K5.D	9297	87	*Corynebacterium / Staphylococcus*	Unoccupied
K6.C	14286	96	*Streptococcus*	Occupied
K6.F	13074	111	*Staphylococcus*	
K6.S	13030	34	*Methyloversatilis*	
K7.A	24971	70	*Propionibacterium*	Occupied
K7.D	37908	76	*Streptococcus*	Occupied
K7.F	31519	102	*Staphylococcus*	
K7.S	11982	22	*Methyloversatilis*	
K8.A	20742	99	*Staphylococcus*	Unoccupied
K8.B	25259	53	*Propionibacterium*	Unoccupied
K8.C	13304	46	*Propionibacterium*	Occupied
K11.D	14429	84	*Streptococcus*	Occupied
K12.C	14764	66	*Propionibacterium*	Unoccupied
K12.E	12120	112	*Acinetobacter*	
K12.F	10391	85	*Streptococcus*	

Table 6.9: Bacterial taxa isolated from Koala ward and their usual habitats.* represents extremophiles.

Taxon	Water	Gut	Respiratory tract	Oral Flora	Faeces	UG tract	Soil/ Plants	Skin	Environment	Food	Mucous membranes	Sewage/ Industry	Unknown	Animals/ Insects
A4													X	
Abiotrophia		X		X		X								
Achromobacter	X						X							
Acidovorax							X							
Acinetobacter							X	X						
Actinobacillus			X	X										
Actinomyces				X			X							
Actinomycetospora							X							
Actinotalea												X		
Adhaeribacter	X						X							
Aerococcus						X								
Aeromicrobium	X								X					
Aggregatibacter			X	X										X
Agrobacterium									X					
Agrococcus							X		X	X				
Alishewanella										X		X		
Alkanindiges												X		
Allobaculum														X
Alliococcus			X (ear)											
Alloscardovia		X		X										

Taxon	Water	Gut	Respiratory tract	Oral Flora	Faeces	UG tract	Soil/ Plants	Skin	Environment	Food	Mucous membranes	Sewage/ Industry	Unknown	Animals/ Insects
Anaerococcus		X		X		X								
Anoxybacillus*	X													
Aquabacterium	X													
Arcanobacterium			X	X		X		X						X
Arthrobacter							X							
Azorhizobium							X							
Azospira							X							
Bacillus									X					
Bacteroides		X			X									
Balneimonas													X	
Bdellovibrio	X						X							
Bifidobacterium		X		X	X	X				X				
Blastomonas	X													
Blautia		X												
Bosea	X						X					X		
Brachybacterium										X				
Brevibacterium							X	X						
Brevundimonas	X													
Brochothrix							X			X				
Buchnera														X
Bulleidia				X										
Burkholderia	X						X		X					
Campylobacter		X												

Taxon	Water	Gut	Respiratory tract	Oral Flora	Faeces	UG tract	Soil/ Plants	Skin	Environment	Food	Mucous membranes	Sewage/ Industry	Unknown	Animals/ Insects
Candidatus Accumulibacter												X		
Candidatus Solibacter							X							
Capnocytophaga				X										
Cardiobacterium			X											
Catenibacterium					X									
Catonella				X										
Cellulomonas							X							
Cellvibrio							X							
Chroococcidiopsis*									X					
Chryseobacterium*	X						X		X					
Citricoccus							X					X		
Clostridium									X					
Comamonas									X					
Coprococcus					X									
Corynebacterium	X						X			X	X			
Curtobacterium							X							
DA101													X	
Deinococcus					X		X		X					
Delftia									X			X		
Dermabacter								X						
Dermacoccus								X				X		
Desulfovibrio	X													

Taxon	Water	Gut	Respiratory tract	Oral Flora	Faeces	UG tract	Soil/ Plants	Skin	Environment	Food	Mucous membranes	Sewage/ Industry	Unknown	Animals/ Insects
Devosia							X							
Dialister			X	X		X								
Dietzia	X						X			X				
Dorea		X			X									
Dyadobacter							X							
Enhydrobacter	X													
Enterobacter	X	X	X				X	X				X		
Enterococcus		X												
Erwinia									X					
Erythrobacter	X													
Escherichia		X												
Eubacterium	X			X			X	X				X		
Exiguobacterium*							X							
Facklamia						X								
Faecalibacterium		X												
Filifactor				X										
Finegoldia				X	X	X	X							
Flavobacterium	X						X							
Friedmanniella												X		
Fusobacterium				X										
Gardenella						X								
Gelidibacter	X													
Gemella		X		X							X			

Taxon	Water	Gut	Respiratory tract	Oral Flora	Faeces	UG tract	Soil/ Plants	Skin	Environment	Food	Mucous membranes	Sewage/ Industry	Unknown	Animals/ Insects
Geobacillus*							X		X	X				
Geodermatophilus							X							
Gillisia	X													
Gluconacetobacter							X							
Gluconobacter							X							
Gordonia	X	X					X		X			X		
Granulicatella				X										
Haemophilus			X											
Haloanella													X	
Helcococcus								X						
Hylemonella	X													
Hymenobacter							X							
Hyphomicrobium	X						X							
Iamia														X
Janibacter									X			X		
Janthiobacterium							X							
Jeotgalicoccus										X				
Kaistobacter									X					
Kingella				X										
Klebsiella	X	X	X		X		X		X	X		X		
Kocuria								X						
Kribbella							X							
Kytococcus								X						
Laceyella							X							

Taxon	Water	Gut	Respiratory tract	Oral Flora	Faeces	UG tract	Soil/ Plants	Skin	Environment	Food	Mucous membranes	Sewage/ Industry	Unknown	Animals/ Insects
Lachnospira														X
Lactobacillus		X				X								
Lactococcus							X			X				
Lautropia				X										
Legionella	X													
Leptochrichia				X										
Leucobacter							X		X	X		X		
Leuconostoc										X				
Limnohabitans	X													
Luteococcus							X							
Luteolibacter							X							
Lysinibacillus							X			X				
Macrococcus														X
Megasphaera		X			X									X
Methylibium	X											X		
Methylobacterium	X						X		X					
Methylosinus	X						X							
Methylotenera									X			X		
Methyloversatilis	X								X					
Microbacterium	X						X			X				
Microbispora							X							
Micrococcus	X						X	X						
Microlunatus							X							
Modestobacter*									X					

Taxon	Water	Gut	Respiratory tract	Oral Flora	Faeces	UG tract	Soil/ Plants	Skin	Environment	Food	Mucous membranes	Sewage/ Industry	Unknown	Animals/ Insects
Mogibacterium				X										
Moraxella			X											
Moryella		X												
Mycobacterium	X	X							X	X				
Mycoplasma								X						
Myroides									X					
Nannocystis							X							
Negativicoccus								X						
Neisseria											X			
Nocardioides							X							
Nostoc	X						X							
Novosphingobium	X						X							
Ochrobactrum							X							
Oligella						X								
Oribacterium			X	X										
Oscillospira									X					
Paenibacillus	X						X		X					
Parabacteroides		X			X									
Paracoccus							X					X		
Parascardovia				X	X									
Patulibacter							X							
Pediococcus										X		X		
Pedobacter	X													
Peptococcus					X	X								
Peptoniphilus						X								

Taxon	Water	Gut	Respiratory tract	Oral Flora	Faeces	UG tract	Soil/ Plants	Skin	Environment	Food	Mucous membranes	Sewage/ Industry	Unknown	Animals/ Insects
Peptostreptococcus		X		X		X		X						
Phaeospirillum	X													
Phenylobacterium							X							
Photobacterium*														
Phycicoccus							X		X					
Planomicrobium									X					
Polaromonas	X													
Pontibacter*									X					
Porphyromonas		X	X	X										
Prevotella				X		X								
Propionicimonas							X							
Propionibacterium						X		X						
Pseudochrobactrum												X		
Pseudoclavibacter							X							
Pseudomonas	X						X	X	X					X
Pseudonocardia							X					X		
Pseudoxanthomonas							X					X		
Psychrobacter*										X				
Rathayibacter							X							
Rheinheimera	X													
Rhodococcus							X		X			X		X
Rhodoplanes	X											X		
Rickettsia							X							X

Taxon	Water	Gut	Respiratory tract	Oral Flora	Faeces	UG tract	Soil/ Plants	Skin	Environment	Food	Mucous membranes	Sewage/ Industry	Unknown	Animals/ Insects
Roseburia		X			X									
Roseococcus									X					
Roseomonas	(X)												X	
Rothia			X	X										
Rubrivivax	X											X		
Ruminococcus		X			X									
Rummeliibacillus							X							
Salinibacterium	X						X							
Sarcina		X					X	X						X
Schlegella*	X											X		
Sejongia	X						X							
Selenomonas				X										X
Serratia	X						X		X					X
Shewanella	X						X			X				X
Simplicispira												X		
Snethia						X								
Sphingobacterium									X					
Sphingobium	X						X							
Sphingomonas	X						X							
Spirosoma	X						X		X					
Sporosarcina							X							
Staphylococcus								X			X			
Stenotrophomonas	X						X							
Streptobacillus														X

Taxon	Water	Gut	Respiratory tract	Oral Flora	Faeces	UG tract	Soil/ Plants	Skin	Environment	Food	Mucous membranes	Sewage/ Industry	Unknown	Animals/ Insects
Streptococcus			X	X				X						
Sulfurospirillum	X													
Sutterella		X			X									X
Tepidimonas*									X				X	
Teracoccus										X				
Tetragenococcus														
Thermicanus							X							
Thermoactinomyces	X						X			X				
Thermus*	X													
Treponema		X		X										X
Trichococcus												X		
Varibaculum														
Veillonella		X		X										
Vibrio	X													X
Virgibacillus							X							
Wautersiella													X	
Weissella										X				
Williamsia									X					
Xanthobacter							X							
Yonghaparkia							X							
Zoogloea	X											X		

17 identified taxa had both human and environmental sources. For 7 taxa, the natural source is unknown, including *Wauterisiella* and *Balneimonas*. Many taxa identified were groups which contain known human pathogens such as *Clostridium*, *Burkholderia*, *Enterobacter* and *Neisseria*.

All surfaces analysed supported the presence of complex microbial communities. A principal component analysis (PCA) was carried out to mathematically test the relationship between bacterial communities from bed space, non-bed space, sink and floor samples. PCA allows a large number of variables (in this case bacterial taxa) to be reduced to smaller numbers, called principal components, whilst maintaining the maximum variance from the data set. This reduction allows complex data sets to be plotted and enables similarities and differences in samples to be visualised (351). Samples that cluster together on a PCA plot show a degree of similarity, in this case they show that they contained a similar taxonomic profile. Bed space samples showed general clustering, as did floor and sink samples. Sink samples were shown to have a clustering pattern that was largely distinct from bed space, non-bed space and floor samples (Figure 6.17).

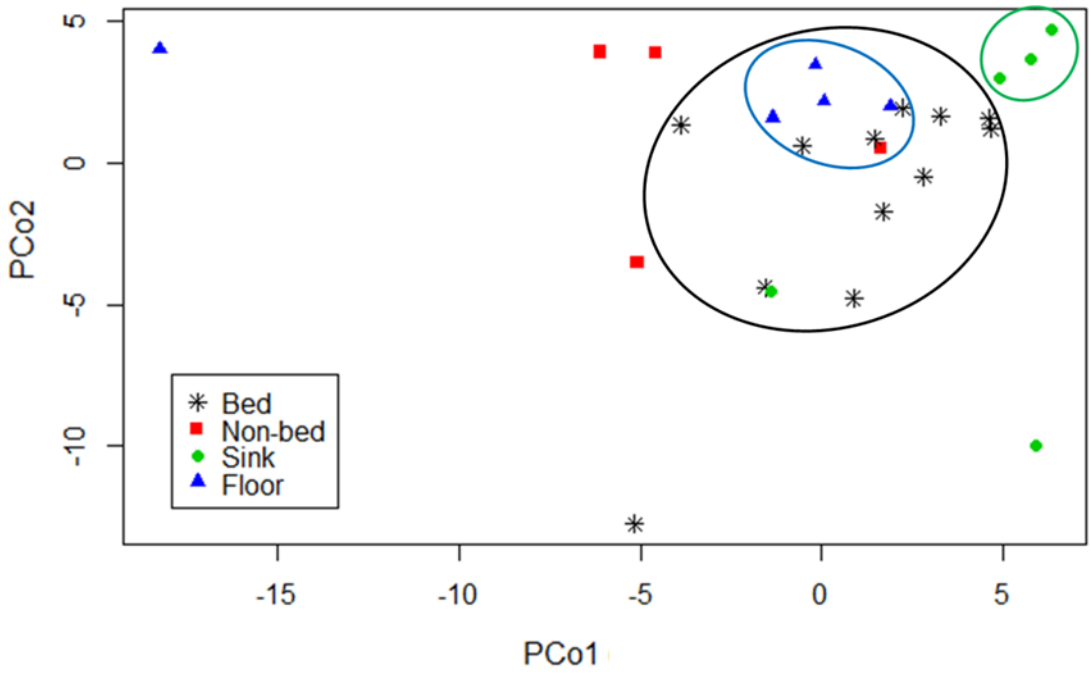


Figure 6.17: Principal Component Analysis, showing clustering of samples with overlaps between bed space and floor samples.

Bed space, non-bed space and floor samples had some overlaps, particularly with reference to the most commonly isolated taxa which made up 1 % or more of total sequence reads (Figure 6.18). Bed spaces had the greatest number of taxa found at > 1 % of sequencing reads than anywhere else on the ward. Bacterial taxa common to bed spaces, non-bed space areas and floors were *Acinetobacter*, *Corynebacterium*, *Enhydrobacter*, *Lactobacillus*, *Neisseria*, *Prevotella*, *Propionibacterium*, *Pseudomonas*, *Rothia*, *Staphylococcus* and *Streptococcus*. More taxa were shared between floors and bed spaces than between floors and non-bed space areas and bed space and non-bed space areas (Figure 6.18).

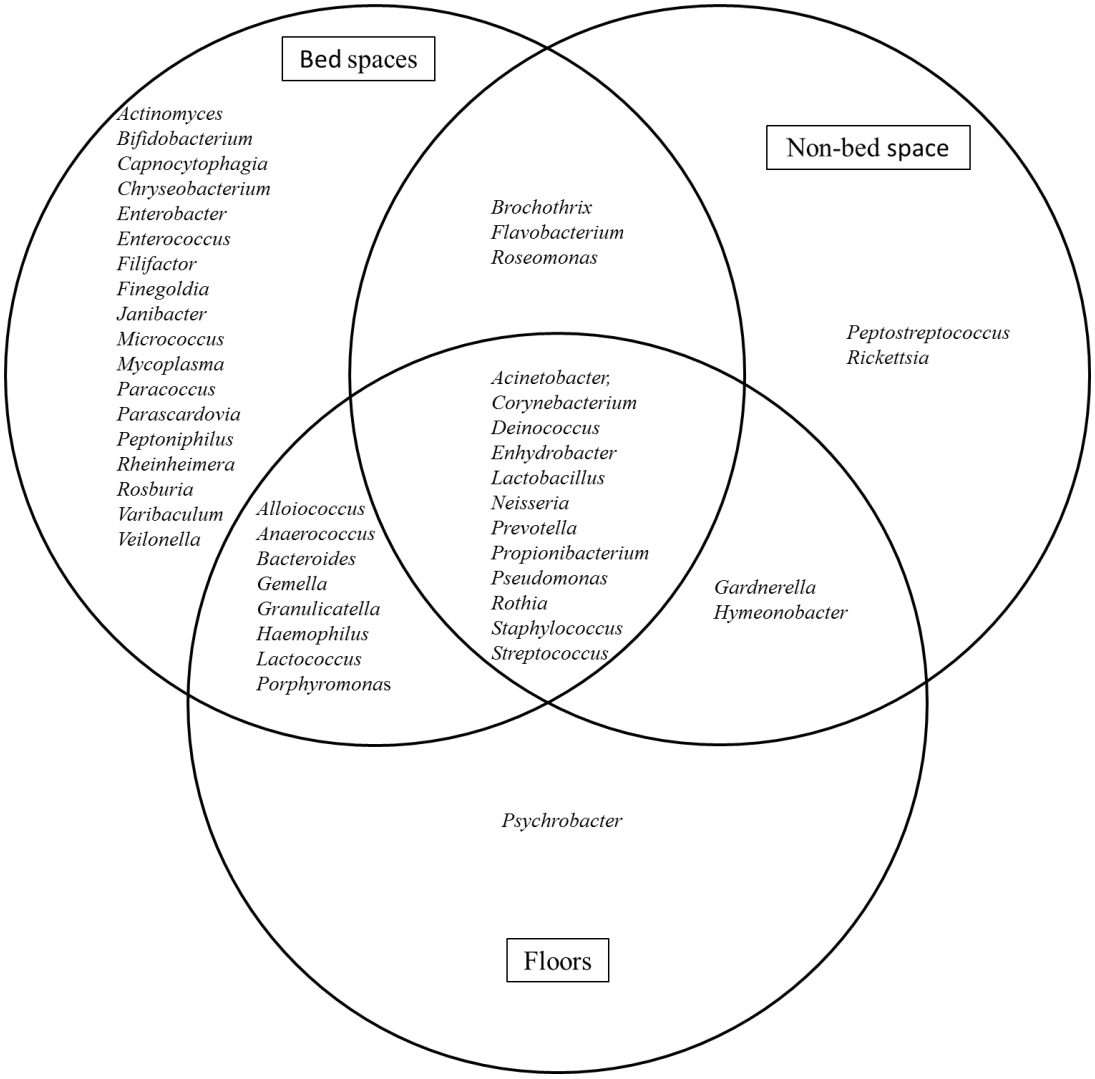


Figure 6.18: Venn diagram showing the taxa forming > 1 % of sequencing reads isolated from 3 different areas of Koala ward. Overlaps indicate taxa isolated from multiple areas.

Bed spaces were largely dominated, in terms of number of sequencing reads, by *Propionibacterium* and this was more pronounced in samples K7.A, K8.C and K12.C (Figure 6.19). *Streptococcus* was dominant in 2 samples K2.A and K11.D and was present in all other samples. *Staphylococcus*, *Corynebacterium*, *Prevotella* and *Acinetobacter* were almost always present in bed space samples (Figure 6.19).

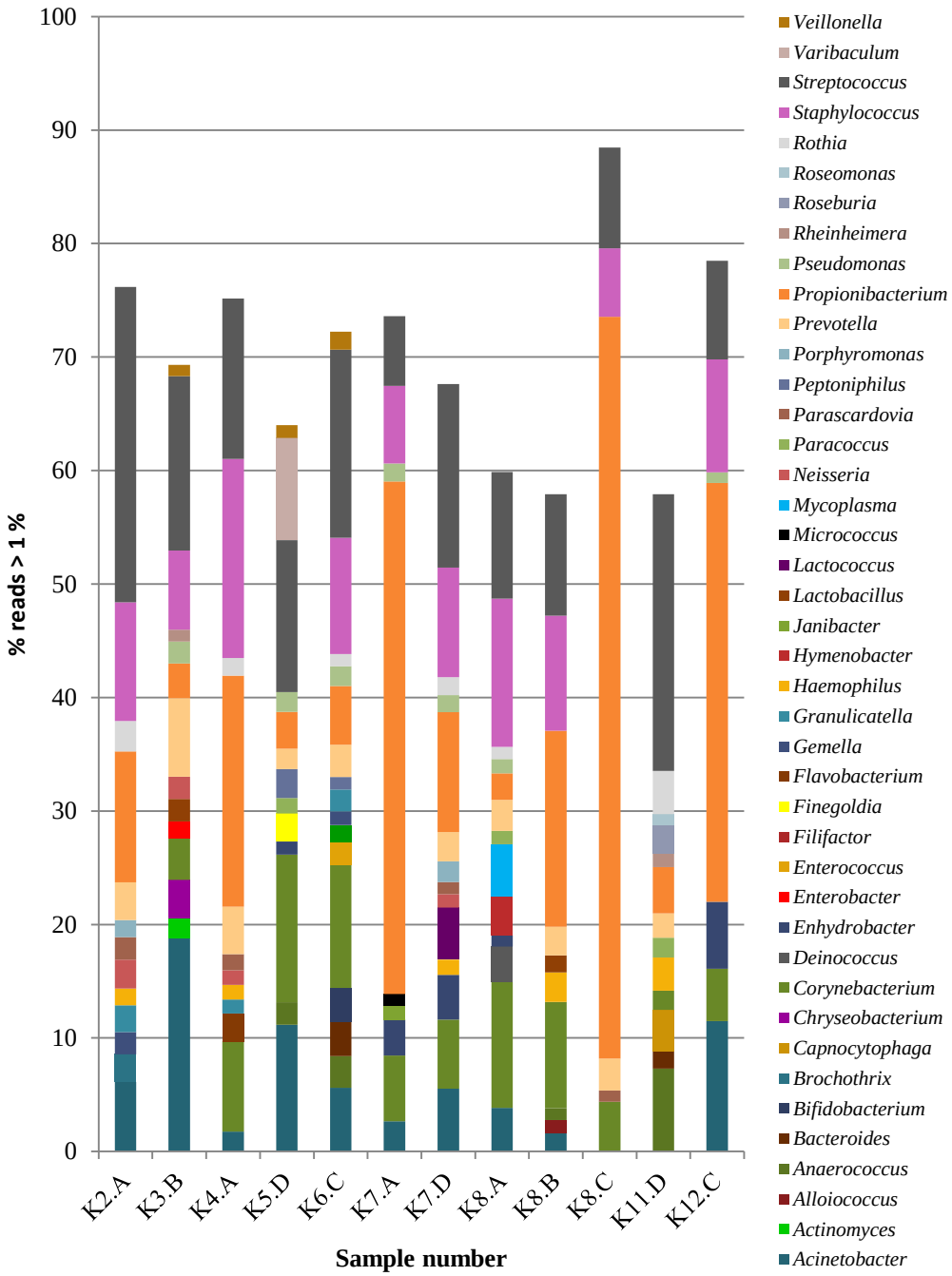


Figure 6.19: Bacterial taxa comprising > 1 % of total sequencing reads in each bed space sample analysed.

Some taxa were each only present in one bed space sample including *Actinomyces*, *Chryseobacterium* and *Enterobacter* in K3.B, *Finegoldia* and *Varibaculum* in K5.D, *Filifactor* and *Enterococcus* in K6.C, *Micrococcus* in K7.A, *Lactococcus* in K7.D and *Mycoplasma*, *Hymenobacter* and *Deinococcus* in K8.A (Figure 6.19). Most of these taxa were only found in bed space samples. The number of taxa representing > 1 % of sequence reads was lowest in sample K8.C and highest in sample K6.C.

Staphylococcus was dominant in 2 non-bed space samples and *Acinetobacter* dominant in one. The remaining sample had no clear dominant genus and *Acinetobacter*, *Staphylococcus*, *Streptococcus* and *Corynebacterium* represented similar numbers of reads (Figure 6.20). *Propionibacterium* was found less frequently than in bed space samples but *Prevotella* was identified in similar proportions. *Peptostreptococcus* was identified from K2.E and K4.E and *Rickettsia* was found in K1.E. Neither genus was found in other areas of the ward.

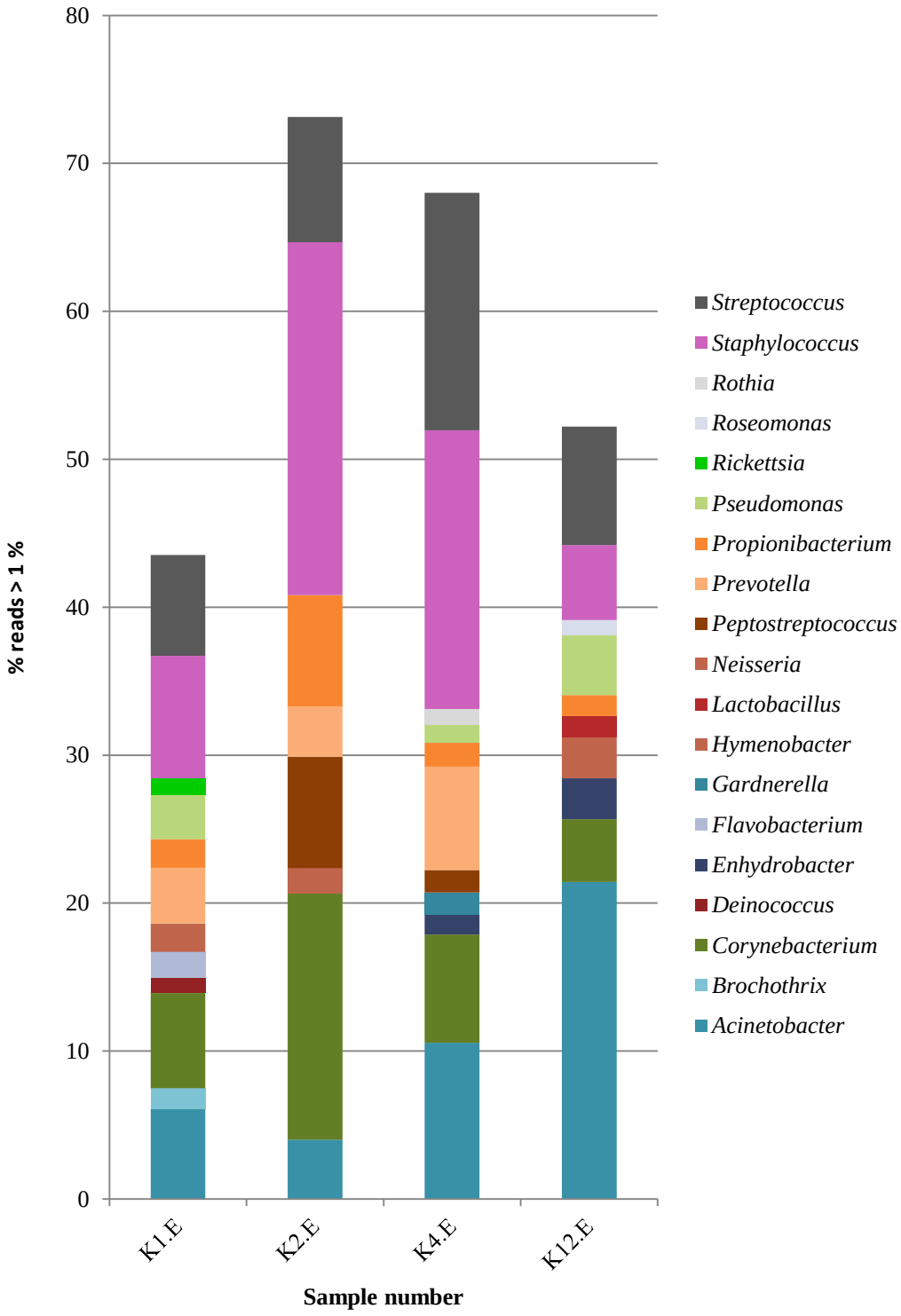


Figure 6.20: Bacterial taxa comprising > 1 % of total sequencing reads in each non-bed space sample analysed.

Staphylococcus was again dominant in most floor samples, with the exception of K12.F, in which *Streptococcus* was dominant in terms of number of sequencing reads (Figure 6.21). *Psychrobacter* was the only genus to be exclusively found in floor samples and was only identified in one sample (K2.F). *Acinetobacter* and *Corynebacterium* were identified in all floor samples, as was *Propionibacterium* but the latter was again present in lower amounts than in bed space samples. *Bacteroides* was only identified from one floor sample (K12.F) and *Granulicatella*, *Haemophilus*, *Neisseria* and *Porphyromonas* were each only identified once in sample K3.F (Figure 6.21).

With the exception of K2.S, in which *Streptococcus* made up the majority of sequencing reads, *Pseudomonas* and *Methyloversatilis* were largely dominant in sink samples (Figure 6.22). Some taxa including *Schlegelella*, *Lautropia*, *Acidovorax*, *Hylemonella* and *Erythrobacter* comprised > 1 % of sequencing reads. These taxa were not found in high proportions in samples from other ward areas, which may account for the tighter clustering of sink samples in comparison to others in the PCA (Figure 6.17).

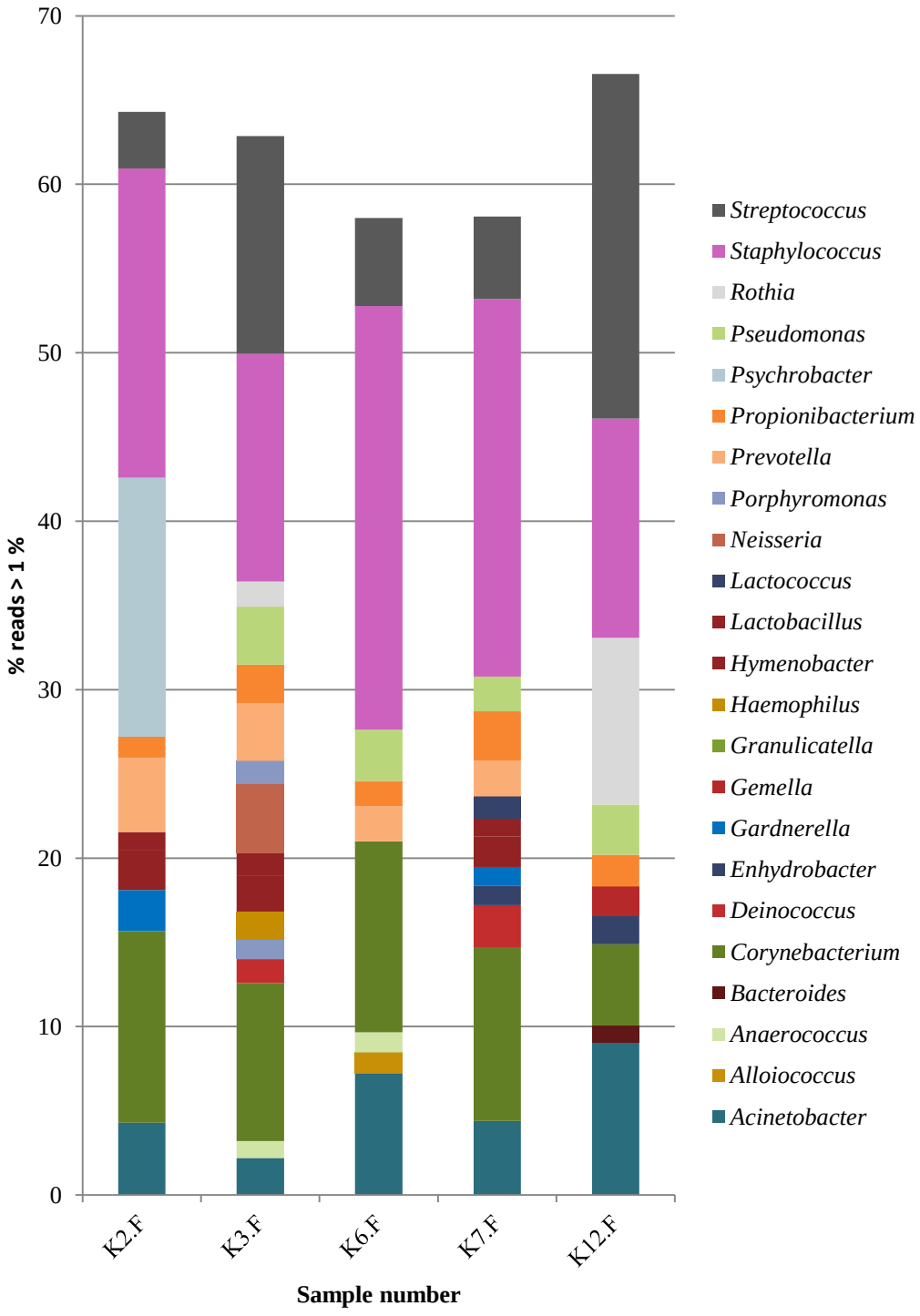


Figure 6.21: Bacterial taxa comprising > 1 % of total sequencing reads in each floor sample analysed.

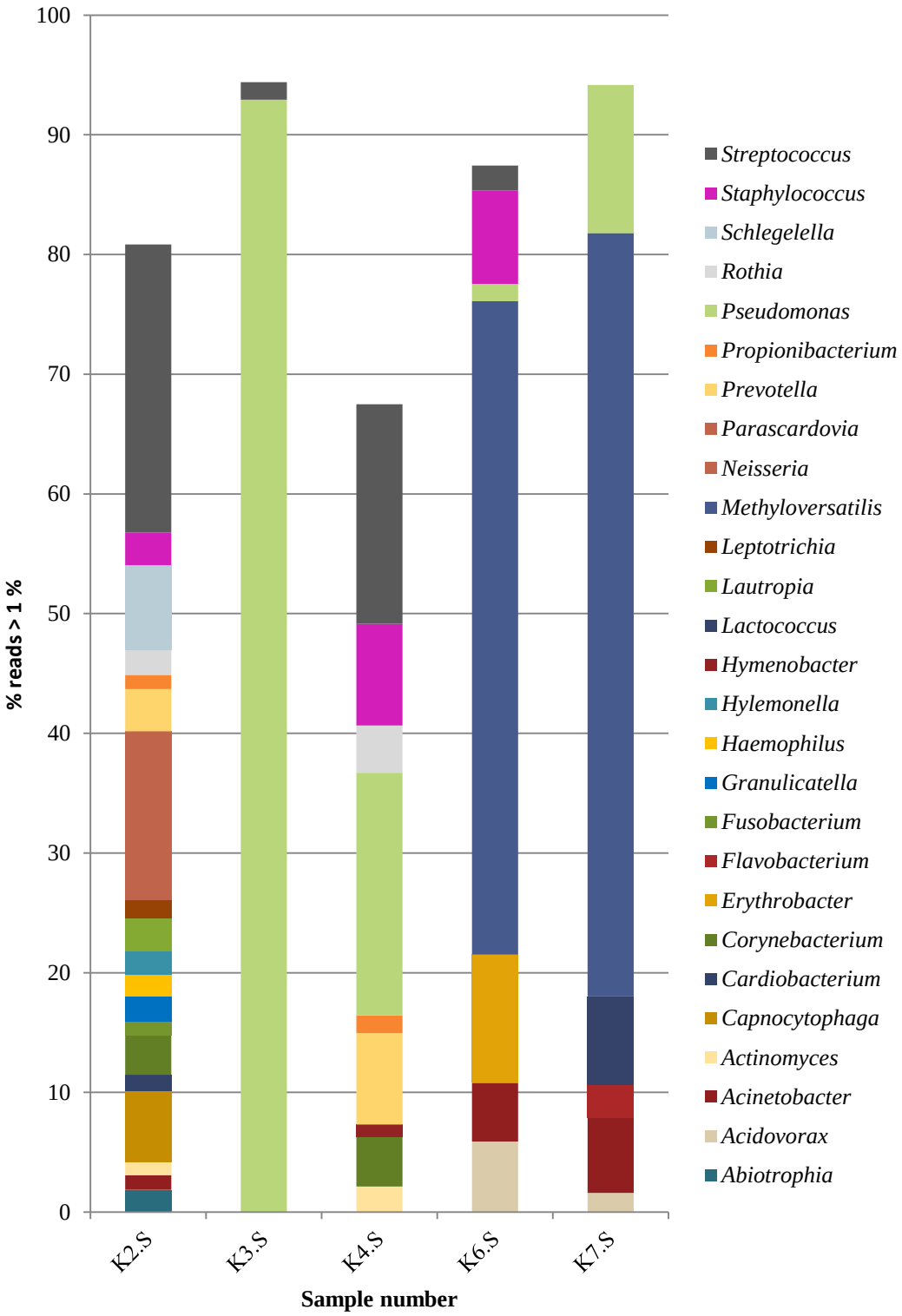


Figure 6.22: Bacterial taxa comprising > 1 % of total sequencing reads in each sink sample analysed.

6.4.6.3 Identification of bacteria associated with nosocomial infection

Figure 6.23 summarises the relative proportions of bacterial genera known to contain species commonly associated with paediatric nosocomial infection that were detected on hospital surfaces throughout the sampling period. *Escherichia* was only identified once in a non-bed space sample in March (K2.E) and made up only 0.06 % of total sequencing reads. *Clostridium* and *Klebsiella* were largely absent from samples and when identified, were present in low proportions of between 0.05 and 0.6 % of total reads. *Enterococcus* was also not commonly found but was identified at bed space C at time point K6 in higher numbers of reads (2 %) than at other time points analysed. *Staphylococcus* was found frequently and in all sample types.

There was significantly more *Staphylococcus* identified on sink samples than floor samples (p = 0.03) and similar proportions were identified from bed space, non-bed space and floor samples. *Streptococcus* was found in all samples and often in lower proportions on sinks, although the sample site did not appear to have a significant impact on the proportion of *Streptococcus* reads identified. *Acinetobacter* was also identified in all samples and was generally at a consistent level. However, K3.B and K12.E had higher proportions of *Acinetobacter* reads than the remaining samples.

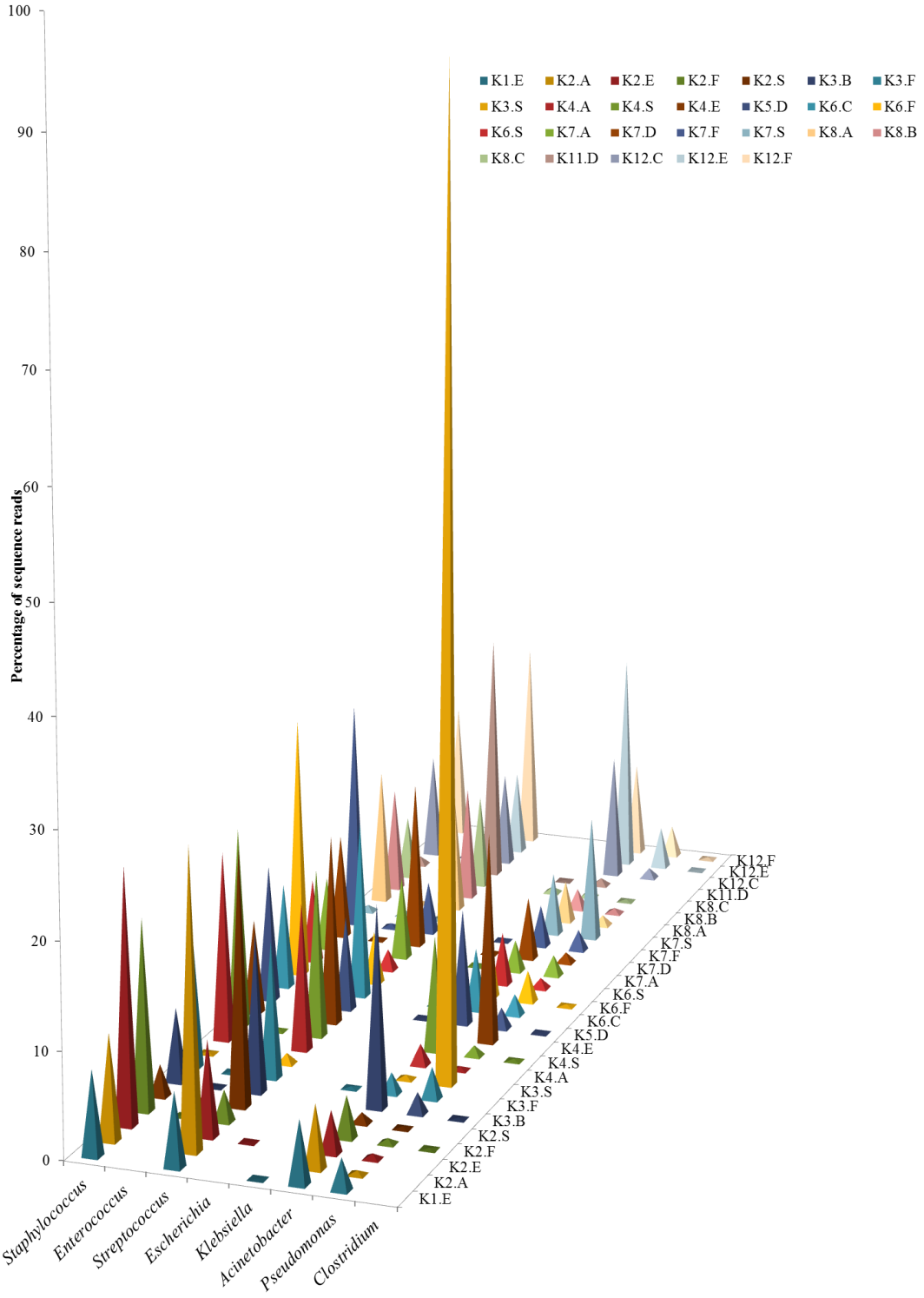


Figure 6.23: Relative proportions of bacterial genera known to contain species commonly associated with paediatric nosocomial infection that were detected on the hospital surfaces throughout the sampling period.

There was no significant difference in sample type and the proportion of *Acinetobacter* identified. *Pseudomonas* was found throughout samples at low levels but greater proportions occurred in all sink samples analysed, with a notable peak in sample K3.S (March), in which 93 % of all sequence reads were identified as *Pseudomonas*. There was a significant difference in the average proportions of *Pseudomonas* observed between the bed space areas and sinks (p = 0.002) and the difference between floors and sinks was almost at a significant level statistically (p = 0.057) but no difference was observed between non-bed space areas and sinks (p = > 0.05).

6.5 Discussion

6.5.1 Bacterial total viable counts

As part of the overall aim to explore the nature and diversity of microorganisms in different indoor environments, this study has analysed the bacterial contamination on a high-dependency children's neurology ward over the course of a year. Monitoring bacterial bioburden is often carried out routinely in healthcare settings to assess the efficacy of cleaning regimes or in response to outbreaks. However, this approach is controversial (13), (45), (163), (205) as it does not take into account specific species and cleaning is a complex issue to address, with conflicting information available regarding its overall contribution to reductions in HCAIs.

Official UK government regulations regarding acceptable levels of microorganisms on hospital surfaces do not currently exist. Levels have been proposed based on food preparation industry standards and in 2003, Malik et al. carried out an audit of surface screening in 4 hospitals and used the cut-off criterion of < 2.5 CFU / cm^2 as a guideline for surface cleanliness (352). They recommended intervention strategies such as further cleaning and investigations should a site fail screening and have more than 2.5 CFU / cm^2 present. They presented no hospital evidence-based reasoning for these guidelines but the limits have since been adopted for further investigative work and even for use on the International Space Station (163), (353). If these criteria were applied to the data collected above, intervention strategies would have to be put in place when a CFU count of 62.5 per TVC contact plate and above was observed. This would mean that over the course of the screening, 26 % of sites would have failed inspection, resulting in vastly increased expenditure on cleaning and other intervention strategies. Close to or over half of sites would have failed at time points K2, K5, K6 and K12, which would likely meet criteria for ward closure if these guidelines were in place. The limit proposed by Malik et al. does not take into account bacterial species; the consequences of *Staphylococcus epidermidis* being present on a surface might be far less than that of the same number or less of pathogenic *E. coli*, for example.

In 2004, SJ Dancer proposed the cut off limit of 5 CFU / cm^2, equating to 125 CFUs per contact plate in the current study. This figure was proposed based on the US Department of Agriculture's limits of bacteria on food-processing equipment (354)

and again, not on any evidence-based hospital screening examples. The hospital environment is dynamic and likely to contain different levels and types of microorganisms to those found in food preparation facilities. However, Dancer has since published articles with other authors and reverted to using the lower cut-off limit, referring to it as a 'standard' (163), (164), (176). As the current study has shown, surfaces are frequently contaminated and at levels above both criteria suggested by Malik and Dancer.

Overall levels of bacteria recovered from TVC plates on the ward were consistent with levels recorded in other areas of the hospital. Lower levels of contamination were observed on high touch areas and higher levels observed on floors and windowsills. This pattern was also seen in the results from Chapter 4 and a recent study conducted at GOSH (un-published data) showed similar patterns of contamination in ICUs, wards and other outpatient's areas. The data from the un-published study combined the analysis of how many times an object was touched, CFU counts and hand washing behaviour and showed that transmission of bacteria to other people likely occurs in shared ward areas. Staff tend to wash hands prior to entering a bed space but often fail to when exiting, meaning that microorganisms from the patient, or objects around them, get transmitted to other areas on the ward. This may explain the often higher TVC counts observed on non-bed space items. In fact, the bed space areas on Koala ward generally had lower TVCs than the other areas, except for at time point K2. This was the first time point when the ward was open to patients and the TVC counts may reflect the fact that staff had only been

working on the ward for 2 weeks and may not have been used to their surroundings and new equipment.

Although no official observational surveys were carried out during the current study, some incidents were noted. One such case was of a patient having a highly-resistant *E. coli* infection but needing the specialist care of the neurology team, so could therefore not be isolated. The patient was nursed on the open ward under strict barrier-nursing conditions. However, it was observed on more than one occasion that nursing staff would attend to the patient wearing gloves and aprons but would often leave the bed space to cross the ward to collect an item, thus potentially spreading bacteria to shared areas.

Bed space TVC counts were shown to be related to the presence of patients with infection control microbial alerts. TVC counts were always lower in the bed space area of a patient with such an alert in place and this could indicate that staff were much more aware of the possibility of contamination of the area and therefore cleaned more frequently and rigorously. This was in contrast to another study conducted at GOSH by Gaudart et al., in which the presence of patients and the presence of patients with an active bacterial infection on Koala ward were shown to be predictors of environmental contamination (45). The study by Gaudart et al. was conducted on 2 intensive care units (ICU) and CFU counts were on average higher than those obtained from the ward in the current study. The ICU environment is more tightly regulated in terms of who can enter and the differences in

contamination levels may indicate differences in cleaning frequency. Regular simple wipe downs of near bed space equipment, as occurred on the open ward in this study appear to reduce levels of bacterial contamination and keep TVCs low.

This study was intended to be an overall long-term analysis of the bacterial numbers and species on Koala ward, with a view to understanding more about the indoor microbiota. As such, staff and patient behaviour, air-flow rates, touch frequencies, exact cleaning times and other factors were not closely monitored. However, these factors may play a role in contributing to environmental bacterial levels and community profiles.

Cleaning cannot be relied on alone to reduce the transmission of microorganisms and can be difficult in practice. Figure 6.24 shows a bed space area on Koala ward when occupied, demonstrating the presence of objects which may contribute to environmental contamination and the potential difficulty in cleaning the area.

The current study shows that despite regular cleaning, surfaces have persistent bacterial contamination. The areas that were regularly cleaned had minimal TVCs, such as patient tables and for the most part, bed rails. When a patient with a microbial alert left a bed space, a level 3 chlorine clean was carried out.

Figure 6.24: A bed space in use on Koala ward, demonstrating potential difficulty in cleaning the space.

This was always apparent (even without discussing with staff) when screening was conducted due to the reduced levels of bacteria in that bed space. The baseline count on Koala ward prior to patients moving in was conducted after a level 3 deep clean had been carried out. The data show low counts in most areas, however the chair arms appeared to perhaps have been overlooked, in that one had a count of 46 CFU, another had 70 CFU and a third had > 200 CFU recovered. Chairs were brand new and a thorough clean was reported to be carried out. A mid-level window ledge and floor also showed high levels of bacterial contamination. This was prior to any patients or staff using the space and indicates that it is difficult to keep items free from bacteria even when an area is not used and has been deep cleaned. K3 screening occurred after a Norovirus outbreak and a level 3 clean had been carried

out. Bed space C was the only occupied area, yet intermediate to high levels of bacteria were found throughout the ward.

Other authors have demonstrated a relationship between relative humidity and temperature and CFU counts (10), (35), (72). However, most of this work has been conducted with regards to air and less is known about how temperature and RH influence surface bacterial counts in indoor environments. Many species of bacteria can survive for months on surfaces, including those associated with HCAIs such as *S. aureus, Klebsiella* spp., *Acinetobacter* spp., *E. coli* and *P. aeruginosa* (67). Low temperatures of 4 – 6 ^{O}C have been associated with increased bacterial survival on experimental surfaces relative to higher temperatures of 18 ^{O}C and above (355), (356). However, increased humidity increases survival rate in the case of *E. coli* (355) and *Acinetobacter* (91) and other bacteria (67). This study did not show any correlation between temperature and CFU counts observed or RH and CFU counts observed. However, bacterial counts were higher during the summer months (K5 and K6). Whilst this does not appear to be related to temperature, it may have been related to windows being opened more frequently during this time, or perhaps even the tendency of people to spend more time outdoors, lying on grass for example. This may have increased the number of bacteria they carried onto the ward. This is however purely speculative but the increase in bacterial contamination during the summer months might be something to be considered when cleaning is carried out. Perhaps a more intensive or frequent cleaning effort could be put into place over these months if a correlation with increased HCAIs is noted. The survival of bacteria

on surfaces is a complex interplay of factors including nutrient and moisture availability, temperature, biofilm formation and others and each surface is likely to have different local conditions.

The mechanisms and relative contributions of different factors to environmental contamination are complex. Despite this, the data presented here demonstrate some patterns and allow some inferences to be made. Overall bacterial counts on Koala ward were low and therefore by inference the presence of pathogens should have also been low. However, the presence of viable but not culturable bacteria and the inability of some species to grow on the type of agar medium used, might mean an underestimation of the true surface contamination present. The presence of a patient did not lead to increased TVCs, in fact when a patient with an infection control alert was present, TVC counts recovered were lower than when a patient without an alert was present. Temperature and RH did not appear to influence TVCs. TVCs cannot provide accurate bacterial identification and are not useful when wanting to assess risk but may provide an overall indication of cleanliness.

6.4.2 *Pseudomonas aeruginosa* isolation

Cultures taken from sinks were analysed for the presence of *Pseudomonas aeruginosa* as it is a recognised nosocomial pathogen and known inhabitant of hospital sinks (14), (86), (170), (167), (320), (357). Breathnach et al. found the presence on sinks of 2 strains of carbapenemase-producing *P. aeruginosa* associated with outbreaks in 2 different UK hospitals (66). Also, the HPA reported 73 hospitals

in the UK with cases of this type of multi-drug resistant *P. aeruginosa* in 2010 – 2011 (358). The results of the *Pseudomonas* screening carried out in this study show the presence of *P. aeruginosa* on Koala ward at the time of screening. However, none of the isolated strains appeared resistant to any antibiotic tested. With the exception of one sink in April (K3), *P. aeruginosa* only appeared in the summer months (K5 - K7) and no culture evidence could be found after August on sinks. Patients were admitted to the ward in July and August with active *P. aeruginosa* infections but appear not to have been the source of sink contamination due to the isolation of the species in April and June. As the patients already had an active infection at the time of admission, it could also be inferred that they did not acquire infection from the sinks on Koala ward.

P. aeruginosa outbreaks are often considered to be a result of poor design or faulty or misused sinks and drainage equipment. The building in which Koala ward is housed was completed and opened for use in 2012 and sink contamination with culturable *P. aeruginosa* appeared 1 month after opening. No issues were reported with sinks during the entire screening process. *Pseudomonas* is ubiquitous in water and appears not to have taken much time to establish a niche in a new sink. The lack of antibiotic resistance in all isolated strains may be an indicator that the contamination was purely environmental. Antibiotic use is the driver of resistance and therefore commonly arises within patients.

6.4.3 NGS analysis of bacterial communities

The use of next-generation sequencing has allowed an insight into the microbial communities present on surfaces on a hospital ward.

The majority of sequences identified from the ward belonged to the phyla Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria. Previous studies using NGS have also found these to be the dominant phyla in a variety of indoor environments (63), (70), (71). Numerous taxa within the phyla observed on the ward are normally associated with humans and form a part of the commensal flora. Many taxa known to contain species responsible for opportunistic infection were also observed, including *Flavobacterium*, *Legionella*, *Stenotrophomonas*, and *Gemella*.

Skin-associated taxa including *Propionibacterium*, *Staphylococcus* and *Streptococcus* were observed in high proportions from all samples. This was not unexpected as it has previously been shown that the main source of microbial particles in indoor environments is the presence of humans (10), (35,48–52) and skin-associated bacteria have been shown to persist on surfaces (13), (359). Other human-associated bacteria were also observed in all samples, including those associated with the oral cavity, mucous-membranes, gut and urogenital tract. *Neisseria* and *Prevotella*, for example, were commonly isolated. Many of these human-associated taxa are anaerobic and would not therefore have been identified using standard culture-based environmental screening methods. Anaerobic bacteria

are of particular concern in hospital environments due to the presence of patients with indwelling devices and the invasive procedures often carried out which can introduce opportunistic pathogens to sites where they may cause infection.

The most commonly observed colonies on TVC plates from bed space items were *Staphylococcus*. Only a small number of cultured isolates were sequenced, however, these did represent the most commonly observed morphotypes and *Propionibacterium* and *Streptococcus* were not identified despite these taxa making up a large proportion of NGS reads obtained. *Staphylococcus* and *Micrococcus* were common, however, indicating that they perhaps out-compete other bacteria which may be present in high numbers on surfaces. *Bacillus* spp. were frequently observed on TVC culture plates, particularly from non-bed space areas such as high windowsills and floors. However, relatively few *Bacillus* sequences were identified through NGS. *Bacillus* spp. were the most frequently isolated bacteria on Tiger ward, the outpatient's area and classroom presented in Chapter 4 and also in similar studies conducted in a child-care facility (360), and school (361). Many *Bacillus* species are known to grow well on blood agar or TSA (362), as used throughout this study. In Chapter 4 and the cited work in schools and child-care facilities, culture was used prior to sequencing and this appears to have led to the overgrowth and therefore over-representation of *Bacillus* spp. in these indoor environments. These findings confirm the need for more molecular-based community analysis work and highlight the importance of not enriching samples prior to sequencing to avoid misrepresentation of community structure.

Bokulich et al. observed similar results to the current work, reporting the presence of high levels of *Streptococcus, Staphylococcus, Neisseria* and Enterobacteriaceae on a neonatal intensive care unit (NICU) (363). They also noted higher abundances of *Acinetobacter* and *Pseudomonas* on non-neonate associated items, which bears some similarity to this study in that *Pseudomonas* was more prevalent on sinks and non-bed space items. *Acinetobacter* however, was found throughout Koala ward. Dunn et al. also found that skin, oral and gut-associated bacteria were dominant in residential environments and found a significant spatial difference with regards to diversity (63). In a study by Hewitt et al., also in a NICU, *Enterobacter, Neisseria, Pseudomonas, Streptococcus* and *Staphylococcus* were all identified from surfaces (364). They also found evidence of other opportunistic pathogens such as *Acinetobacter, Clostridium* and *Legionella* and that NICU bacterial diversity is similar to that of other environments, with human skin being a primary contributor to surface contamination. The same again was found by Oberauner et al. when looking at microbial communities in an ICU (365).

It appears that microbial communities in a variety of indoor spaces share similar patterns and these tend to be dominated largely by bacteria normally-associated with humans. However, the consequences of the presence of potentially pathogenic bacteria in hospital and healthcare environments may be far greater than in other locations.

The presence of gut and urogenital tract-associated bacteria implies a lack of compliance with hand washing guidelines or inadequate cleaning after nappy-changing or bed-pan use. Gut-associated bacterial taxa including *Bacteroides*, *Faecalibacterium*, *Blautia* and *Enterobacter* were found in all samples and are of particular concern in healthcare environments. A large proportion of gut-associated bacteria are Gram negative and these bacteria have a natural resistance to some antibiotics. In an environment where there is added selective pressure due to the prescribing of antibiotics, resistance genes can be transferred, leading to a spread of resistance between species. *Bacteroides* spp. can be significant clinical pathogens and can cause bloodstream infection (BSI) and wound infections and have been shown to be highly resistant to many antibiotic classes (366). *Enterobacter* spp. are also frequently associated with BSI (367).

A large number of different environmental taxa were identified on the ward in all samples. Environmental bacteria may often be considered to be non-pathogenic but certain taxa can be of concern, particularly in a hospital environment. *Gordonia* spp. are environmental bacteria which have recently been shown to be increasingly related to human infection, causing a wide range of clinical diseases including BSI and brain abscesses (368). *Ochrobactrum* spp. are soil and water-dwelling organisms but have been identified as the causal agents of bacteraemia in neonates (369), (370) and septic arthritis in an immunocompetent adult (371). *Pseudoclavibacter* spp. are, again, soil bacteria but have been found in clinical samples (372) and implicated in wound-infection (373). It may be that bacterial taxa considered to be only

environmentally-associated and non-pathogenic may play a greater role in human infection than previously thought. The ability to detect these uncommon indoor taxa through NGS may allow greater insight into their roles as pathogens.

In contrast to microbial communities identified by other authors in other indoor areas, there appeared to be a relatively even spread of taxa throughout the ward, with no significant difference in the number of taxa isolated from bed space, non-bed space and floor and samples. Sinks on Koala ward, however had a lower number of bacterial taxa than other areas and a distinct profile. In a study investigating the bacterial communities within public toilet facilities, it was found that there was a significantly more diverse bacterial community associated with the floor than other areas of the facility (70). This was not seen on Koala ward and may be due to the much more frequent and rigorous cleaning carried out in hospital environments. Areas in homes and kitchens which were subject to less cleaning have been shown to have higher levels of bacterial diversity (63), (71). Flores et al. reported the presence of soil-associated bacteria in higher proportions on floors than other surfaces and this was also observed in the Koala ward samples. Whilst all ward floor samples had *Staphylococcus* or *Streptococcus* identified in the largest proportion, other taxa such as *Acinetobacter* and *Psychrobacter* were present in higher proportions than in samples from other areas of the ward. This change in dominant taxa would perhaps indicate that the bottom of shoes bring in bacteria from the outside environment.

TVC counts were shown to be lower when patients were present with microbial alerts, perhaps indicating more frequent and effective cleaning of these bed spaces. Only 2 samples from bed spaces with patients with microbial alerts were successfully amplified and sequenced (K6.C and K8.C). This may mean that less DNA was present in other cases, leading to non-amplification. The patient in bed space C at time point K6 had active bacterial infections with *Enterococcus*, *Klebsiella*, and *Stenotrophomonas maltophilia*. *Enterococcus* sequences were found at 2 % of total reads for this sample. This genus was identified in 9 of 12 bed space samples that were sequenced but the average proportion of reads observed was only 0.1 %. This is much lower than observed in the bed space of the patient who had an active *Enterococcus* infection. *Stenotrophomonas* sequences made up 0.06 % of reads for this bed space which was slightly lower than the average of 0.07 % for all other bed space samples and the same applied to *Klebsiella*. The floor and sink samples were also sequenced for this time point and neither showed elevated levels of any of the 3 genera related to the patient's infections. This may reflect differences in ability to recover or sequence Gram negative species. The patient in bed space C at K8 had active infections with *P. aeruginosa*, *Prevotella* and *Serratia marcescens*, however higher proportions of sequence reads were not recorded for any of the related genera. Due to the lack of samples from bed spaces of patients with active infection, it cannot be accurately determined if they contributed to contamination of their near or wider environment.

The presence of *Pseudomonas* on sink samples was confirmed through culture and biochemical analysis. Sequence analysis showed no *Pseudomonas* sequences at time point K2 but at time point K3, *Pseudomonas* made up 93 % of the total sequence reads for that sample (Table 6.10).

Table 6.10: Number of reads observed for *Pseudomonas* sp. from all sink samples and the proportion of total reads they represent

Sample	Reads	% total reads
K2.S	0	0
K3.S	14049	93
K4.S	9062	21
K6.S	187	1.44
K7.S	1489	12

This corresponds to the culture data in that the first time point when *P. aeruginosa* was isolated from a sink was also K3. K5, K6 and K7 were also culture positive for *P. aeruginosa*. The sink sample from K5 failed to amplify but *Pseudomonas* sequences were observed at K6 and K7, although in lower proportions than K3 and K4. At the 2 former time points, *Methyloversatilis* was the dominant genus, accounting for 55 % and 64 % of sequence reads. *Methyloversatilis* was first isolated from the sediment of Lake Washington in 2004. The type species is *M. universalis* and it has been shown to be resistant to ampicillin, erythromycin, chloramphenicol, trimethoprim / sulfamethoxazole (SXT), vancomycin and penicillin (374). There is no available literature regarding the presence of *Methyloversatilis* in indoor environments, or its potential for pathogenicity. However, it is a Gram negative bacterium with antibiotic resistance characteristics and was found not only on sink samples, but also floor and non-bed space items on the hospital ward at many

sampling points, so may be of future concern. Other taxa not found commonly throughout the ward were observed in sink samples including *Schlegelella, Hylemonella, Erythrobacter, Lautropia* and *Acidovorax.* The three former taxa are associated with water (375), (376), (377). However, *Erythrobacter* spp. tend to be associated with sea water and in this case, the taxonomic identification may have failed to correctly identify this isolate. *Erythrobacter* is however a member of the Sphingomonadaceae family which have members known to be associated with hospital tap water (378).

Despite the difficulties encountered with library preparation for NGS analysis, useful sequence data were recovered. This work has however confirmed difficulties faced by other authors in assessing microbial communities in hospital environments due to the very small amount of DNA that can be isolated from these surfaces (379). This may explain the lack of studies published from these environments and the lack of long-term studies, as missing data points make time series analysis impossible.

There appears to have been no relationship between TVC counts and ability to amplify DNA for NGS. Very low TVC counts were recovered from the ward at the K1 baseline-sampling time point and only one of these samples (K1.E) yielded enough DNA to sequence. At the K2 time point, average ward CFUs increased and more samples were sequenced. However, intermediate TVC counts were recovered from bed space A (K2.A) and this sample was able to be sequenced, yet bed space D had higher TVC counts and was not able to be amplified. Highest TVC counts were

recorded at time points K5 and K6 but only one of 7 samples from K5 could be amplified and only 3 from K6. Total CFU levels were low again after these two time points until K12 when they increased to a higher level, yet only some samples from K7, K8 and one from K11 amplified. Inhibition of each sample was tested for using the internal positive control qPCR assay and inhibited samples were excluded, so the reason for poor or no amplification of samples remains unknown. The barcoded primers along with linkers and adapters for NGS are 62 bp long for the forward primer and 68 bp for the reverse, which might account for some of the difficulty in amplification of target sequence DNA, especially at low target concentration.

Despite the knowledge that PCR amplification with greater than 30 cycles can significantly increase the numbers of chimeric sequences obtained, leading to questionable identification, work has been published using over this number of cycles, presumably due to the low amount of DNA present (363), (365). These studies, and no doubt others who do not report full PCR conditions, used a nested PCR strategy to first amplify the whole 16S rRNA gene, followed by amplification of the target region within the gene to add barcodes. The current study however, was aimed at obtaining sequence identification that was as accurate as possible and therefore only used a maximum of 30 cycles of PCR amplification. As such and by using the identification algorithms present in the QIIME software, it can be assumed that bacterial identification is as correct as possible. This may have come at the detriment of losing a large proportion of samples and not being able to analyse the full time series as initially intended, however the results obtained can be relied upon

as an accurate as possible representation of bacterial taxa present at time of sampling.

The lack of samples amplifying from the baseline time point meant that sequence data obtained at subsequent time points could not be compared and an understanding of if and how microbial communities changed from baseline could not be established. However, the non-bed space sample from K1 was analysed and showed no difference in the number of taxa identified compared to non-bed space samples from later time points. Although, at the baseline time point, there was no clearly dominant genus present, with *Streptococcus*, *Staphylococcus*, *Acinetobacter* and *Corynebacterium* making up similar proportions of total sequencing reads. This changed at subsequent time points with *Staphylococcus* becoming dominant in terms of numbers of sequencing reads. This probably reflects the increase in human activity on the ward after K1. No large differences were observed in other sample types between time points. Bed space samples sometimes had greater proportions of *Propionibacterium* than at other time points (K7.A, K8.C and K12.C) however; this was likely due to the presence of an individual with higher numbers of this genus present on their skin. The same applies to floor samples when peaks in taxa such as *Psychrobacter* at K2 and *Rothia* at K12 likely indicate an outside source. Although taxa identified could not be fully analysed as a function of time, time did not appear to alter the microbial communities present.

The > 97 % similarity level cut-off for identification used in this study was chosen as it is currently the standard for studies investigating microbial communities (63), (71), (226), (363), (364), (365), (380), (381). The cut-off is chosen to identify taxa which contain closely related members and does not identify to species-level. This enables the dataset to be more manageable in downstream analysis. The method used also has some inherent problems when it comes to species-level identification. For example, a number of bacterial genera do not have enough heterogeneity in the 16S rRNA gene region to be able to establish a difference and assign a species. *Streptococcus mitis* and *S. oralis*, for example, show 99 – 100 % similarity in the V5 – V7 region of the 16S rRNA gene, therefore cluster together as 1 species (214). It is also not always possible to assign a taxonomic lineage due to the extensive nature of reference databases and the presence of incomplete and un-verified sequences (214). The naïve Bayesian classification method used in this study and by others uses sequences from the Ribosomal Database Project (RDP) to identify bacterial taxa in accordance with the standard Bergey system of bacterial taxonomy. This has been shown to identify full and partial-length 16S rDNA sequences of 400 bp or more to the genus level with an accuracy of approximately 88.7 % (220). However, the accuracy when attempting to identify species is reduced to approximately 25 %. The lack of species resolution makes it impossible to predict the pathogenicity of any identified bacteria, however, a high proportion of taxa identified do contain species that are known human pathogens, including *Acinetobacter*, *Stenotrophomonas*, *Flavobacterium* and *Escherichia*. The presence of these and other bacterial genera in a hospital environment could be a concern and previous studies have shown that

when comparing bacterial taxa found on surfaces and hospital-acquired infections over the same time period in the same area, there was a correlation (365).

Another issue surrounding correct identification is the hypervariable region of the 16S rRNA gene chosen for use. The V6 region (as used in this work) has been described as the most variable of the 16S rRNA hypervariable regions, and its use gives high levels of discriminatory power (214). Currently, however, there is no single primer set that can be used for NGS that is capable of identifying all known bacteria which may lead to misevaluation of the diversity present (147), (222). Further knowledge may have been gained from this study by the use of more than one primer set for NGS, however, the cost was prohibitive for this work. The primers used in this study were chosen as they had previously been shown to be useful in identifying a broad range of bacterial isolates (214).

Another general limitation of the technique is that PCR cannot detect bacterial replication competence. This may lead to an over-estimation of risk as a proportion of bacterial sequences identified may have originated from non-viable organisms. However, the presence of bacterial nucleic acid means that the organism must have been present at some point in time and may very well have been capable of infection or colonisation. GOSH infection control policy indicates that presence of microbial nucleic acids from nominated pathogenic species after cleaning, indicates a cleaning failure and that there may still be potential for infection. Also, non-viable organisms

can still transfer genetic material to others and therefore still pose a threat should they carry drug-resistance genes.

The diversity and composition of bacterial communities observed on Koala ward is likely driven by several factors, including the frequency of cleaning, the number of people in the environment and the sources of bacteria introduced into the environment. Time series analysis could not be conducted; however, diversity in samples did not appear to alter significantly between samples from different time points. This indicates that the bacterial communities within this ward were fairly stable. Occasional local differences were observed at bed spaces, perhaps indicating the presence of a person carrying higher numbers of certain taxa.

Bacterial communities showed some clustering when a PCA was carried out indicating that bed space samples were similar in diversity as were floor samples. Non-bed space items did not cluster together, indicating that they often had different community compositions which may reflect differences in microorganisms carried onto the ward by different people and outside sources. There was not a large distinction between clusters of these three types of sample, indicating an overlap of community members but sinks had a distinct clustering pattern, likely due to the higher numbers of water-associated bacteria present. The clustering patterns observed may have been more pronounced if more samples had been included. It is difficult to draw solid conclusions from small data sets such as this but general trends could be seen from the data. Human-associated bacteria were found in high

proportions throughout the ward with some taxa such as *Propionibacterium* being more frequently found in the bed space areas. *Staphylococcus*, *Streptococcus*, *Prevotella*, *Acinetobacter* and *Corynebacterium* were ubiquitous.

This study highlights that despite frequent cleaning and a relatively regulated environment, distinct and diverse microbial communities are present on surfaces on a paediatric hospital ward. Overall contamination levels appear low when using standard culture methods and do not reflect the diversity of bacteria truly present. Levels of contamination peak in the summer months for reasons unknown and not related to temperature or RH but tend to be lower around patients with microbial alerts as issued by infection control teams. Bacterial communities tend to contain fewer members than other indoor environments and appear to be fairly stable with respect to composition but do contain taxa which may be related to human pathogens. Skin-associated bacteria are the most commonly identified groups in this environment, as has been found with other indoor environments but environmental bacteria are also common. These groups may be of clinical consequence in a hospital environment and their presence should not be over-looked.

7.Summary and conclusions

7.1 Project background

Microorganisms are resilient, can utilise diverse energy sources and can survive in an astounding range of ecological niches, from the extreme heat and lack of light found at hydrothermal vents (382), to the sub-zero temperatures and lack of nutrients in Arctic permafrost (383). It is therefore unsurprising that they have been able to adapt to the man-made built environment and establish connections to the health and disease states of the people who inhabit these places. The contribution of microorganisms present in indoor environments to disease in particular, has been known for many years. Florence Nightingale was a staunch advocate of window ventilation to hospital wards and homes in order to prevent illness and also recognised the role of fomites in the transmission of disease. Writing in *'Notes on Nursing: What it is, and what it is not'* in 1859 (384), she commented:

"For a sick room, a carpet is perhaps the worst expedient which could by any possibility have been invented. If you must have a carpet, the only safety is to take it up two or three times a year, instead of once. A dirty carpet literally infects the room. And if you consider the enormous quantity of organic matter from the feet of people coming in, which must saturate it, this is by no means surprising."

As technology has advanced, the scientific community has been better able to understand the mechanisms and microorganisms responsible for the contamination noted by Nightingale and others. The microbial communities in indoor environments have, until relatively recently, only been understood with regards to what could be cultured on agar plates. This methodology, whilst having provided useful information in the past, cannot always be relied upon to capture the entirety of a microbial community due to the presence of viable but not culturable members and those which have fastidious growth requirements (15–17). The agar culture method also fails to take into consideration the presence of viruses, which require complex, time-consuming and expensive identification processes.

Molecular methods, in particular PCR, have been used to gain a greater understanding of indoor microbial communities but due to previous technical limitations, such as the need for cloning and sequencing, have often failed to capture the full diversity of microorganisms present. Also, as viruses do not contain a universal gene target such as the 16S rRNA gene found in bacteria and Archaea, molecular methods for virus identification have tended only to focus on specific species. This means that limited knowledge is available regarding the viral component of indoor ecosystems. In addition, very little comparative work is available directly comparing microbial ecosystems in different environments.

At the time of commencement of the current study, NGS was beginning to be applied as a rapid, relatively cheap and accessible method for microbial community

analysis. It had previously been beyond the capacity of small laboratories and many research projects. As such, very few investigations had beied out into the microbial communities of indoor spaces.

In view of the above outline, this project aimed to fill three major gaps in the available literature; to determine:

1. If levels of microbial contamination and community composition were different in healthcare and educational settings.
2. If viruses could be isolated from the air and from surfaces in a healthcare environment and what factors might influence this.
3. If microbial numbers and community composition changed over the course of a year on a new hospital ward and if the causes and consequences of any observed changes could be identified.

These aims were achieved through the sampling of various indoor environments using a combination of standard monitoring techniques such as TVC culture plates and molecular methods such as qPCR and NGS.

7.2 Summary of main findings

Through using a combination of culture and molecular techniques, various different bacteria were found to be present in different environments that are frequented by children. Overall bacterial contamination in terms of TVCs was lower in hospital

environments than in a classroom and this was likely due to the increased cleaning frequency on the ward and outpatient's waiting area. Items located > 1.5 m from the ground tended to be more contaminated than lower items, with the exception of floors, which were found to have amongst the highest TVC counts in all 3 locations. Items that were frequently touched often had low TVCs and this was probably due to the fact that they were obvious targets for cleaning.

Small numbers of bacterial taxa were found in all 3 environments initially sampled and *Bacillus* was the most commonly isolated genus from the classroom and outpatient's area. Tiger ward also had a high proportion of *Bacillus* present but *Staphylococcus* was the most frequently observed genus. *Pseudomonas* also appeared to be more prevalent on the ward than the outpatient's area and classroom. The low numbers of taxa observed compared to NGS studies was due to the methods used to isolate bacteria from these environments, demonstrating that culture-based techniques do not capture full diversity. Due to the small number of bacteria isolated however, species resolution was possible in some cases and resulted in the identification of potentially pathogenic organisms in all 3 environments. *E. cloacae* was isolated from all 3 environments and *A. baumanii* was isolated from Tiger ward. As a result of the findings presented in Chapter 4, infection control teams at GOSH reviewed the use and cleaning frequency of the outpatient's waiting area in order to prevent the potential transmission of infection to susceptible patients.

Chapter 5 presents data which show that viruses can be isolated from an indoor environment. A number of different viral nucleic acids were identified on surfaces in an outpatient's clinic at GOSH. Adenovirus DNA was ubiquitous and often present in very high copy numbers. Despite not being able to determine the consequences of this in terms of infection risk, the presence of such high copy numbers of a known viral pathogen was a concern. Other viral nucleic acids were isolated but with lower frequency and this could be due to the fact that patients with NV and RV, for example, may not have attended the clinic if they had acute illness. That being said, viral nucleic acids from these species were still identified and the only potential source would have been the presence of an infected person as the viruses identified do not grow outside of human cells.

The use of TTV as a viral marker of contamination was also investigated and results indicated that it might be a more useful marker than TVCs, which are often used. As reported by other authors in studies investigating environmental virus contamination (44), door handles were frequently contaminated with virus nucleic acids. This part of the study was a proof-of-concept investigation and showed that viral nucleic acids are present on surfaces and in the air of clinical environments and may indicate the presence of infectious viruses which may be a risk to patient and staff health.

Investigation of the bacterial contamination of a high-dependency children's ward over the course of a year showed that the ward environment in this case, was generally quite clean in terms of TVCs. Peaks in contamination levels were recorded

2 weeks after the opening of the ward and in summer months. This may indicate that cleaning regimens could be intensified over the course of the summer, should these higher counts relate to incidence of infection. TVC counts however do not provide extensive information regarding bacterial identification and can therefore not be directly related to risk. Indeed, some taxa, such as *Bacillus* and *Micrococcus* appeared common to TVC plates but were not present as high proportions of sequencing reads obtained when NGS was carried out. Numerous taxa were also identified by sequencing that would fail to grow on TVC plates and may be a risk to patients.

Bacterial contamination was less when a patient with a microbial alert was present but patients did not appear to alter the amount of contamination otherwise. This implies that staff members were aware of the potential of transmission from surfaces when a patient had a known infection and took steps to reduce it. However, it has previously been reported that patients are at greater risk of acquiring an infection if a previous room occupant had that infection (162). This may be due to the contamination of non-bed space items and these items were found to have higher overall TVC counts in the current study. It may also not apply to open wards which may be more frequently cleaned, or where surfaces are wiped down frequently by staff.

The number of bacterial taxa isolated using NGS in Chapter 6 was far greater than the culture and sequencing method used in Chapter 4. Also, it indicated that the

dominant taxa in terms of sequencing reads were usually human-associated when considering bed space, non-bed space and floor samples. The findings were similar to published studies from hospital and other indoor environments.

Despite frequent cleaning, viruses and complex bacterial communities persist on indoor surfaces. It has been demonstrated by other authors that frequent cleaning reduces the abundance of potentially pathogenic bacterial taxa on hospital surfaces (363) and also leads to lower diversity present (62), (70). This may be of consequence where human pathogens may accumulate and out-compete non-pathogenic bacteria. However, cleaning is an important part of hospital infection control strategies and it may reduce the incidence of some nosocomial infections. Less is known about the importance of bacterial communities in other indoor environments but this study has shown that TVCs can be high and potentially pathogenic species can be isolated from classrooms. These environments do not have the same strict cleaning regimes as healthcare environments but the presence of bacteria may not be as concerning due to the lack of people with underlying conditions.

One of the greatest difficulties in preventing infections and outbreaks is identifying the sources and transmission routes of pathogenic microorganisms. It is known that the presence of humans is the main contributing factor to the type and level of microbial contamination observed in indoor environments. The results presented in this thesis demonstrate that human-associated bacteria dominate most surfaces

within a paediatric hospital ward and that patterns, although not as distinct as in other indoor areas, can be seen with regards to which taxa occupy which areas. Sinks have a different pattern of bacterial dominance, with water-associated taxa being more prevalent. Although the sources of nosocomial outbreaks are often difficult to ascertain, the presence of microorganisms on surfaces indicates that these may be potential reservoirs of infection. These findings may imply that more of an emphasis should be placed on interventions that reduce the initial contamination, rather than trying to deal with it once it is established.

Due to the ever-increasing threat from antibiotic-resistant microorganisms, low compliance with hand-hygiene methods, persistence of bacteria in indoor environments that have been designed to reduce their presence and issues surrounding cleaning materials and strategies, more control mechanisms need to be discovered. Some authors propose that simple methods such as returning to natural ventilation and allowing sunlight into buildings might reduce nosocomial infection (385). Whilst it is true that natural ventilation reduces risk of airborne infection, this strategy is not enough for the prevention of nosocomial infection from surface sources. Other authors suggest more technological solutions may be imminent. With the use of NGS technologies, indoor environments could be rapidly screened and rather than sterilize everything, targeted solutions could be produced, leaving potentially beneficial bacteria in place (386).

The decreasing cost of high-throughput NGS could possibly see its use in routine monitoring of healthcare environments. It could be a valuable tool to measure the 'normal' profile of an area, if DNA amplification issues could be circumvented. By monitoring any changes away from this normal baseline, an early-warning system for preventing the transmission of HCAIs could be put in place. It would also allow the effects of interventions to be studied in much greater detail. However, it is clear from the presented research and the lack of published data providing long-term information, that the techniques and methodologies are not always straightforward, especially when dealing with low concentration DNA samples, as may be obtained from a relatively clean hospital environment. The time taken to obtain data from NGS is comparable to culturing with a single run on a MiSeq taking approximately 39 hours. However, the analysis of data is still time consuming and requires specialist knowledge to interpret. Therefore, NGS technologies do not currently have the capacity to provide rapid, real-time information applicable to high turnover clinical environments

7.3 Limitations of the study

The amplicon sequencing technique used as part of this study to investigate microbial communities is a powerful and rapid tool for high throughput microbial identification. Despite this, however, it still has numerous shortcomings. The 'Operational Taxonomic Unit' is described as organisms sharing > 97 % similarity in their 16S rRNA gene sequence and translates to genus-level identification in this and other studies. This cut-off is an arbitrary value and does not provide species

resolution. Relatively short read lengths coupled with the limitations of extraction methods, PCR biases, limitations of databases, chimeric sequences and other perhaps unknown factors, make results difficult to interpret at species level and this is therefore rarely reported, with most researchers only identifying to genus level.

Although the methods used are not currently sensitive enough to provide species-level identification for all bacteria present and cannot therefore identify the presence of known pathogens, the prevalence of skin and gut-associated bacteria throughout the indoor environment is a concern. Pathogens frequently found in the gut and on the skin may be transmitted via surfaces and cause colonisation or infection. This lack of taxonomic resolution makes it impossible to differentiate pathogens and non-pathogens from the same genus. Findings could be supplemented with other identification techniques such as species-specific qPCR, for example but this negates the purpose of NGS high throughput sequencing. As technology advances and more sophisticated solutions become available, this limitation will likely become less of an issue. The current development of longer read lengths from Illumina of 2 X 300 bp and the introduction of 3rd-generation sequencing technologies such as those from Pacific Biosciences may enable greater resolution from microbial communities. It may also be of interest to consider the importance of microbial community profiles and how various species might interact with each other in this particular environment. Diversity studies taking into account the functional potential of bacteria present may indicate pathogenicity or the transfer of virulence elements between bacteria (387). They may also be able to differentiate live, VBNC and dead

cells. Low diversity taxa (< 1 % of total) have been shown to be the most metabolically active in the gut (388), perhaps this may also apply to hospital environments, explaining why known pathogens may be present in low abundance but remain an issue in terms of clinical disease. Of course, this is speculation and could only be determined with large-scale functional studies, or 'metatranscriptomics', which was beyond the scope of this study. Also, these types of investigations cannot be carried out until metagenomic studies have been first applied.

Another limitation of this technique is the use of the 16S rRNA gene itself. As previously mentioned, despite this gene being used for the majority of published community analysis studies, it may lack discriminatory power for certain taxa. In addition, there is evidence that horizontal gene transfer and recombination of the 16S rRNA gene can occur and may lead to incorrect identification of bacteria (389), (390). Horizontal gene transfer is the exchange of DNA between different species and has previously been thought not to have occurred with the 16S rRNA gene due to its vital and stable function (390). Despite these issues, the 16S rRNA gene is currently the most frequently used and is arguably, the best tool available for microbial community analysis.

DNA extraction methods have been shown to influence the number of microbial taxa identified from NGS samples (347), (391). PCR bias is also a major limitation of the technique in that it can lead to erroneous identification due to amplification of errors

throughout the cycling process (346), (392). The DNA extraction technique used in this study appeared to provide the best results when comparing different methods but may have contributed to the low DNA concentrations obtained. However, the method is widely used and performed well with test samples. DNA amplified well with standard 16S rRNA gene primers and issues only occurred when carrying out barcode PCR, which could not have been foreseen when choosing a DNA extraction method. Swabbing surfaces using cotton swabs was shown to recover < 10 % of test bacteria in Chapter 3. This indicates that the sampling method is also a limiting factor in DNA recovery and alternative methods may provide a better representation of bacteria present. The cotton swabs used in this study are however common to environmental sampling studies, including those which use NGS (70), (71), (364). Perhaps as methods develop and the use of NGS becomes more commonplace, the focus of these studies will become the development of sampling techniques and pre-sequencing processing in order to provide more accurate data.

The study of bacterial microbiomes through NGS and high-throughput sequencing is relatively new and at the time of commencing this project, no work had been published regarding its use in indoor environments. The Illumina MiSeq was not commercially available and standard protocols have still yet to be published and accepted. As is the case with any new technology, the rapid evolution of methods and race for discovery often lead to later understanding of flaws in methodology and analysis. As understanding grows, better methods are developed and understanding is deepened. This project was undertaken when little was known about the microbial

communities within indoor environments, with reference to NGS and has therefore been able to identify not only some interesting insights but also some limitations to the techniques used. It therefore provides a useful basis for further work to explore the hospital microbiome, in particular, in greater depth.

The main limitation of this study was the lack of amplification of a number of samples collected from the ward of the course of the year. The reasons for this remain unknown but appear to be due to a combination of factors which may include the presence of low amounts of DNA coupled with chemicals which may affect the sequencing reaction and possibly the length of the barcode primers themselves. In contrast to other environmental samples such as soil, the overall numbers of microorganisms on hospital surfaces is very low. This may be compounded by the regular cleaning occurring in healthcare environments. This appears to lead to low amounts of DNA being able to be isolated from surfaces which leads to difficulty in community analysis. However, despite not all samples being successfully amplified, the project has been able to address a number of the initial aims. One of the main aims of this project was to determine the 'baseline' composition of microbial communities present on the ward and see if and how this changed with time. Due to the difficulty in amplification of DNA, this aim was not able to be achieved. However, enough samples were sequenced to see that the general community structure does not appear to change significantly over time. Microbial communities in this environment appear to be fairly stable in terms of taxa present and those which are observed in greatest frequency.

7.4 Future work

This study, while significantly contributing to knowledge surrounding the indoor microbiota, has raised some questions that may be addressed with future work. Some of these questions may be explored through the following research directions:

1. Further testing of environmental sampling, DNA extraction methods and PCR amplification to attempt to overcome difficulties with samples.
2. The comparison of 16S rRNA gene regions in their ability to identify bacterial taxa.
3. The use of specific qPCR panels to further investigate the species of bacteria present and molecular typing to relate them to patient infection.
4. The use of newer NGS technologies with longer read lengths and alternative PCR-free methods for microbial community analysis.
5. The larger-scale sampling of different areas to detect virus nucleic acids
6. The direct comparison of different indoor environments using NGS.
7. The analysis of the function of microorganisms identified.

7.5 Conclusion

This study intended to explore different aspects of the indoor microbiome in different environments using a variety of techniques, to highlight the differences and similarities between them and discuss the findings in regards to furthering scientific knowledge.

Microorganisms were present in all locations sampled and were detectable by a variety of techniques ranging from laboratory culture to specific qPCR and cutting-edge NGS. Each of these techniques provides different information that when combined, gives a great insight into the microbiome of an indoor space. However, the meaning of the findings is still hard to decipher. It is clear that microorganisms are present in indoor environments in spite of various solid strategies to remove them and prevent their spread, such as cleaning, hand hygiene and engineering design. What that means in terms of risk is difficult to assess without full knowledge of species, antibiotic resistance profiles and patient infection information.

Related publications

1. D'Arcy, N., Canales, M., Spratt, D.A., Lai, K.M. Healthy schools: standardisation of culturing methods for seeking airborne pathogens in bioaerosols emitted from human sources. *Aerobiologia.* 2012

2. Nikki D'Arcy, Elaine Cloutman-Green, Ka Man Lai, Dimitrious Margaritis, Nigel Klein, David A Spratt. Potential exposure of children to environmental microorganisms in indoor healthcare and educational settings. *Indoor and Built Environment.*

3. Jean Gaudart, Elaine Cloutman-Green, Serge Guillas, Nikki D'Arcy, John C. Hartley, Vanya Gant, Nigel Klein. Healthcare Environments and Spatial Variability of Healthcare Associated Infection Risk: Cross-Sectional Surveys. 2013. *PLoS ONE* 8(9): e76249.

Under review

1. Louise Pankhurst, Elaine Cloutman-Green, Melissa Canales, Nikki D'Arcy, John Hartley. Routine Monitoring of Adenovirus and Norovirus within the Healthcare Environment. Submitted to *Journal of Hospital Infection*

2. Nikki D'Arcy, Elaine Cloutman-Green, Nigel Klein, David A. Spratt. Environmental viral contamination in a paediatric hospital: implications for infection control. Submitted to *American Journal of Infection Control*

3. Elaine Cloutman-Green, Nikki D'Arcy, David Spratt, John C Hartley, Nigel Klein. How Clean is Clean - Is a New Microbiological Standard Required? Submitted to *American Journal of Infection Control*

References

1. Rook G. The hygiene hypothesis and the increasing prevalence of chronic inflammatory disorders. *Trans R Soc Trop Med Hyg*. 2007 Nov;101(11):1072–4.

2. Ege MJ, Mayer M, Schwaiger K, Mattes J, Pershagen G, van Hage M, *et al*. Environmental bacteria and childhood asthma. *Allergy*. 2012 Dec;67(12):1565–71.

3. Humphries C. Indoor Ecosystems. *Science*. 2012 335;(6069):648–50.

4. Kembel SW, Jones E, Kline J, Northcutt D, Stenson J, Womack AM, *et al*. Architectural design influences the diversity and structure of the built environment microbiome. *ISME*. 2012 Aug;6(8):1469–79.

5. Agranovski IE, Safatov AS, Borodulin AI, Pyankov OV, Petrishchenko VA, Sergeev AN, *et al*. New personal sampler for viable airborne viruses: feasibility study. *J Aerosol Sci*. 2005 May;36(5-6):609–17.

6. Knight R, Jansson J, Field D, Fierer N, Desai N, Fuhrman JA, *et al*. Unlocking the potential of metagenomics through replicated experimental design. *Nat Biotechnol.* 2012 Jun;30(6):513–20.

7. Sahlberg B, Gunnbjörnsdottir M, Soon A, Jogi R, Gislason T, Wieslander G, *et al*. Airborne molds and bacteria, microbial volatile organic compounds (MVOC), plasticizers and formaldehyde in dwellings in three North European cities in relation to sick building syndrome (SBS). *Sci Total Environ*. 2013 Feb 1;444:433–40.

8. Tischer CG, Heinrich J. Exposure assessment of residential mould, fungi and microbial components in relation to children's health: achievements and challenges. *Int J Hyg Environ Health*. 2013 Mar;216(2):109–14.

9. Beggs CB, Kerr KG, Noakes CJ, Hathway EA, Sleigh PA. The ventilation of multiple-bed hospital wards: review and analysis. *Am J Infect Control*. 2008 May;36(4):250–9.

10. Cole EC, Cook CE. Characterization of infectious aerosols in health care facilities: an aid to effective engineering controls and preventive strategies. *Am J Infect Control*. 1998 Aug;26(4):453–64.

11. Weber DJ, Rutala WA, Miller MB, Huslage K, Sickbert-Bennett E. Role of hospital surfaces in the transmission of emerging health care-associated pathogens: norovirus, *Clostridium difficile*, and *Acinetobacter* species. *Am J Infect Control*. 2010 Jun;38(5 Suppl 1):S25–33.

12. Dancer SJ. Importance of the environment in meticillin-resistant *Staphylococcus aureus* acquisition: the case for hospital cleaning. *Lancet Infect Dis*. 2008 Feb;8(2):101–13.

13. Dancer SJ. The role of environmental cleaning in the control of hospital-acquired infection. *J Hosp Infect*. 2009 Dec;73(4):378–85.

14. Bernard MC, Lanotte P, Lawrence C, Goudeau A, Bernard L. Air contamination around patients colonized with multidrug-resistant organisms. *Infect Control Hosp Epidemiol*. 2012 Sep;33(9):949–51.

15. Amann RI, Ludwig W, Schleifer KH. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev*. 1995 Mar;59(1):143–69.

16. Head I, Saunders J, Pickup R. Microbial Evolution, Diversity, and Ecology: A Decade of Ribosomal RNA Analysis of Uncultivated Microorganisms. *Microb Ecol*. 1998 Jan;35(1):1–21.

17. Ritz K. The plate debate: cultivable communities have no utility in contemporary environmental microbial ecology. *FEMS Microbiol Ecol*. 2007 Jun;60(3):358–62.

18. Bibby K, Viau E, Peccia J. Viral metagenome analysis to guide human pathogen monitoring in environmental samples. *Lett Appl Microbiol*. 2011 Apr;52(4):386–92.

19. Carducci A, Verani M, Lombardi R, Casini B, Privitera G. Environmental survey to assess viral contamination of air and surfaces in hospital settings. *J Hosp Infect*. 2011 Mar;77(3):242–7.

20. Nazaroff WW. Norovirus, gastroenteritis, and indoor environmental quality. *Indoor Air*. 2011 Oct;21(5):353–6.

21. Tuladhar E, Hazeleger WC, Koopmans M, Zwietering MH, Beumer RR, Duizer E. Residual viral and bacterial contamination of surfaces after cleaning and disinfection. *Appl Environ Microbiol*. 2012 Nov;78(21):7769–75.

22. Goyal SM, Anantharaman S, Ramakrishnan MA, Sajja S, Kim SW, Stanley NJ, *et al*. Detection of viruses in used ventilation filters from two large public buildings. *Am J Infect Control.* 2011 Oct;39(7):e30–8.

23. Yáñez MA, Nocker A, Soria-Soria E, Múrtula R, Martínez L, Catalán V. Quantification of viable *Legionella pneumophila* cells using propidium monoazide combined with quantitative PCR. *J Microbiol Methods.* 2011 May;85(2):124–30.

24. Mayer KH, Opal S. Unusual nosocomial pathogens. I*nfect Dis Clin North Am*. 1989;3(4):833–99.

25. Alvarez S, Shell CG, Woolley TW, Berk SL, Smith JK. Nosocomial infections in long-term facilities. *J Gerontol.* 1988 Jan;43(1):M9–17.

26. Cebular S. Community-acquired pneumonia in immunocompromised patients. Opportunistic infections to consider in differential diagnosis. *Postgrad Med*. 2003;113(1):65–6.

27. National Institutes of Health [Internet]. Human Microbiome Project. [cited 2013 Apr 17]. Available from: http://commonfund.nih.gov/hmp/

28. Koren O, Spor A, Felin J, Fåk F, Stombaugh J, Tremaroli V, *et al*. Human oral, gut, and plaque microbiota in patients with atherosclerosis. *Proc Natl Acad Sci USA*. 2011 Mar 15;108 Suppl 4592–8.

29. Earth Microbiome Project [Internet]. [cited 2013 Apr 17]. Available from: http://www.earthmicrobiome.org/

30. Di Giulio M, Grande R, Di Campli E, Di Bartolomeo S, Cellini L. Indoor air quality in university environments. *Environ Monit Assess*. 2010 Nov;170(1-4):509–17.

31. Jo WK, Seo YJ. Indoor and outdoor bioaerosol levels at recreation facilities, elementary schools, and homes. *Chemosphere*. 2005;61(11):1570–9.

32. Tsai FC, Macher JM. Concentrations of airborne culturable bacteria in 100 large US office buildings from the BASE study. *Indoor Air*. 2005 Jan;15 (Suppl 9):71–81.

33. Starlander G, Melhus Å. Minor outbreak of extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* in an intensive care unit due to a contaminated sink. *J Hosp Infect*. 2012 Oct;82(2):122–4.

34. Newman M. Neonatal intensive care unit: reservoirs of nosocomial pathogens. *West Afr J Med*. 2002;21(4):310–2.

35. Obbard JP, Fang L-S. Airborne concentrations of bacteria in a hospital environment in Singapore. *Water, Air Soil Pollut*. 2003;144:333–41.

36. Bartlett KH, Kennedy SM, Brauer M, van Netten C, Dill B. Evaluation and determinants of airborne bacterial concentrations in school classrooms. *J Occup Environ Hyg*. 2004 Oct;1(10):639–47.

37. Seino K, Takano T, Nakamura K, Watanabe M. An evidential example of airborne bacteria in a crowded, underground public concourse in Tokyo. *Atmos Environ*. 2005 Jan;39(2):337–41.

38. Augustowska M, Dutkiewicz J. Variability of Airborne Microflora in a Hospital Ward. *Ann Agric Environ Med*. 2006;13(April):99–106.

39. Kim KY, Kim CN. Airborne microbiological characteristics in public buildings of Korea. *Build Environ*. 2007 May;42(5):2188–96.

40. Ferroni A, Werkhauser-Bertrand A, Le Bourgeois M, Beauvais R, Vrielynck S, Durand C, *et al*. Bacterial contamination in the environment of hospitalised children with cystic fibrosis. *J Cyst Fibros*. 2008 Nov;7(6):477–82.

41. Al-Shahwani MF. Bacterial distribution analysis of the atmosphere of two hospitals in Ibb, Yemen. *East Mediterr Heal J*. 2005;11(5-6):1115–9.

42. Okten S, Asan A. Airborne fungi and bacteria in indoor and outdoor environment of the Pediatric Unit of Edirne Government Hospital. *Environ Monit Assess*. 2012 Mar;184(3):1739–51.

43. Rettberg P, Fritze D, Verbarg S, Nellen J, Horneck G, Stackebrandt E, *et al*. Determination of the microbial diversity of spacecraft assembly, testing and launch facilities: First results of the ESA project MiDiv. *Adv Sp Res.* 2006 Jan;38(6):1260–5.

44. Wojgani H, Kehsa C, Cloutman-Green E, Gray C, Gant V, Klein N. Hospital door handle design and their contamination with bacteria: a real life observational study. Are we pulling against closed doors? *PLoS One*. 2012 Jan;7(10):e40171.

45. Gaudart J, Cloutman-Green E, Guillas S, D'Arcy N, Hartley JC, Gant V, *et al*. Healthcare environments and spatial variability of healthcare associated infection risk: cross-sectional surveys. *PLoS One.* 2013 Jan;8(9):e76249.

46. Ortiz G, Yagüe G, Segovia M, Catalán V. A study of air microbe levels in different areas of a hospital. *Curr Microbiol*. 2009 Jul;59(1):53–8.

47. Qian J, Hospodsky D, Yamamoto N, Nazaroff WW, Peccia J. Size-resolved emission rates of airborne bacteria and fungi in an occupied classroom. *Indoor Air*. 2012 Aug;22(4):339–51.

48. Hospodsky D, Qian J, Nazaroff WW, Yamamoto N, Bibby K, Rismani-Yazdi H, *et al*. Human occupancy as a source of indoor airborne bacteria. *PLoS One*. 2012 Jan;7(4):e34867.

49. Kalogerakis N, Paschali D, Lekaditis V, Pantidou A, Eleftheriadis K, Lazaridis M. Indoor air quality—bioaerosol measurements in domestic and office premises. *J Aerosol Sci*. 2005 May;36(5-6):751–61.

50. Clark RP, de Calcina-Goff ML. Some aspects of the airborne transmission of infection. *J R Soc Interface.* 2009 Dec 6;6(October):S767–82.

51. Tham KW, Zuraimi MS. Size relationship between airborne viable bacteria and particles in a controlled indoor environment study. *Indoor Air*. 2005 Jan;15 Suppl 9:48–57.

52. Ambroise D, Greff-Mirguet G, Gorner P, Fabries J, Hartemann P. Measurement of indoor viable airborne bacteria with different bioaerosol samplers. *J Aerosol Sci*. 1999;30(Suppl. 1):699–700.

53. Pittet D, Dharan S, Touveneau S, Sauvan V, Perneger T V. Bacterial contamination of the hands of hospital staff during routine patient care. *Arch Intern Med.* 1999 Apr 26;159(8):821–6.

54. Holt JG. Bergey's Manual of Determinative Bacteriology. 9th ed. Maryland: Williams & Williams; 1994.

55. Goh I, Obbard JP, Viswanathan S, Huang Y. Short Communication Airborne Bacteria and Fungal Spores in the Indoor Environment. *Acta Biotechnol*. 2000;20(1):67–73.

56. Health Protection Agency. Health Protection in the 21st Century. London; 2005.

57. Henriques-Normark B, Normark S. Commensal pathogens, with a focus on *Streptococcus pneumoniae*, and interactions with the human host. E*xp Cell Res*. 2010;316(8):1408–14.

58. Abut LI, Apan T, Otlu B, Calişkan A, Durmaz R. The characteristics of nasopharyngeal *Streptococcus pneumoniae* in children attending a daycare unit. *New Microbiol*. 2008 Jul;31(3):357–62.

59. Todar K. Todar's Online Textbook of Bacteriology [Internet]. 2008 [cited 2013 Apr 20]. Available from: http://textbookofbacteriology.net/

60. Quality of Urban Air Review Group. Airborne Particulate Matter in the United Kingdom. Birmingham; 1996.

61. Lee S-H, Lee H-J, Kim S-J, Lee HM, Kang H, Kim YP. Identification of airborne bacterial and fungal community structures in an urban area by T-

RFLP analysis and quantitative real-time PCR. *Sci Total Environ*. 2010 Feb 15;408(6):1349–57.

62. Zuraimi MS, Tham KW. Indoor air quality and its determinants in tropical child care centers. *Atmos Environ*. 2008 Mar;42(9):2225–39.

63. Dunn RR, Fierer N, Henley JB, Leff JW, Menninger HL. Home Life: Factors Structuring the Bacterial Diversity Found within and Between Homes. P*LoS One.* 2013;8(5):e64133.

64. Gilbert Y, Veillette M, Duchaine C. Airborne bacteria and antibiotic resistance genes in hospital rooms. *Aerobiologia*. 2010 Feb 11;26(3):185–94.

65. Peccia J, Milton D, Reponen T, Hill J. A Role for Environmental Engineering and Science in Preventing Bioaerosol-Related Disease. *Environ Sci Technol.* 2008;42(13):4631–7.

66. Breathnach AS, Cubbon MD, Karunaharan RN, Pope CF, Planche TD. Multidrug-resistant *Pseudomonas aeruginosa* outbreaks in two hospitals: association with contaminated hospital waste-water systems. *J Hosp Infect*. 2012 Sep;82(1):19–24.

67. Kramer A, Schwebke I, Kampf G. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *BMC Infect Dis*. 2006 Jan;6(130).

68. Monteil H, Harf-Monteil C. Aerobic gram-negative bacilli: newer nosocomial pathogens. *Int J Antimicrob Agents*. 1997 May;8(4):217–31.

69. Ali S, Moore G, Wilson APR. Effect of surface coating and finish upon the cleanability of bed rails and the spread of *Staphylococcus aureus. J Hosp Infect.* 2012 Mar;80(3):192–8.

70. Flores GE, Bates S, Knights D, Lauber CL, Stombaugh J, Knight R. Microbial Biogeography of Public Restroom Surfaces. *PloS One*. 2011;6(11):e628132.

71. Flores GE, Bates ST, Caporaso JG, Lauber CL, Leff JW, Knight R, *et al*. Diversity, distribution and sources of bacteria in resedential kitchens. *Environ Microbiol.* 2013;5(2):588–96.

72. Wu Y, Yao M. Effects of microwave irradiation on concentration, diversity and gene mutation of culturable airborne microorganisms of inhalable sizes in different environments. *J Aerosol Sci.* 2011 Nov;42(11):800–10.

73. Petti S, De Giusti M, Moroni C, Polimeni A. Long-term survival curve of methicillin-resistant *Staphylococcus aureus* on clinical contact surfaces in natural-like conditions. *Am J Infect Control*. 2012 Dec;40(10):1010–2.

74. Yau YH, Chandrasegaran D, Badarudin A. The ventilation of multiple-bed hospital wards in the tropics: A review. *Build Environ*. 2011 May;46(5):1125–32.

75. Escombe AR, Moore DAJ, Friedland JS, Evans CA, Gilman RH. Natural ventilation for the prevention of airborne contagion. *PLoS Med*. 2007;4(2):e195.

76. Maron PA, Mougel C, H. Lejon DP, Carvalho E, Bizet K, Marck G, *et al*. Temporal variability of airborne bacterial community structure in an urban area. *Atmos Environ.* 2006 Dec;(40):8074–80.

77. Matthias-Maser S, Peters K, Jaenicke R. Seasonal Variation of Primary Biological Aerosol Particles. *J Aerosol Sci.* 1995;26(Suppl. 1):S545–6.

78. Hathway EA, Noakes CJ, Sleigh PA, Fletcher LA. CFD simulation of airborne pathogen transport due to human activities. *Build Environ.* 2011 Dec;46(12):2500–11.

79. Chapman A, Witkop C, Escobar J, Schlorman C, DeMarcus L, Marmer L, *et al*. Norovirus outbreak associated with person-to-person transmission. *Med Surveill Mon Rep.* 2011;18(11).

80. Otter JA, Yezli S, French GL. The role played by contaminated surfaces in the transmission of nosocomial pathogens. *Infect Control Hosp Epidemiol*. 2011 Jul;32(7):687–99.

81. Shaughnessy MK, Micielli RL, DePestel DD, Arndt J, Strachan CL, Welch KB, *et al*. Evaluation of hospital room assignment and acquisition of *Clostridium difficile* infection. *Infect Control Hosp Epidemiol*. 2011 Mar;32(3):201–6.

82. Datta R, Platt R, Yokoe DS, Huang SS. Environmental cleaning intervention and risk of acquiring multidrug-resistant organisms from prior room occupants. *Arch Intern Med.* 2011 Mar 28;171(6):491–4.

83. Drees M, Snydman DR, Schmid CH, Barefoot L, Hansjosten K, Vue PM, *et al*. Prior environmental contamination increases the risk of acquisition of vancomycin-resistant enterococci. *Clin Infect Dis*. 2008 Mar 1;46(5):678–85.

84. Fierer N, Liu Z, Rodríguez-Hernández M, Knight R, Henn M, Hernandez MT. Short-term temporal variability in airborne bacterial and fungal populations. *Appl Environ Microbiol*. 2008 Jan;74(1):200–7.

85. Noris F, Siegel JA, Kinney KA. Evaluation of HVAC filters as a sampling mechanism for indoor microbial communities. *Atmos Environ*. 2011 Jan;45(2):338–46.

86. Bhalla A, Pultz NJ, Gries DM, Ray AJ, Eckstein EC, Aron DC, *et al*. Aquisition of Nosocomial Pathogens on Hands After Contact With Environmental Surfaces Near Hospitalized Patients. *Infect Control Hosp Epidemiol.* 2004;25(2):164–7.

87. Kim SJ, Si J, Lee JE, Ko G. Temperature and humidity influences on inactivation kinetics of enteric viruses on surfaces. *Environ Sci Technol.* 2012 Dec 18;46(24):13303–10.

88. Traoré O, Springthorpe VS, Sattar SA. A quantitative study of the survival of two species of *Candida* on porous and non-porous environmental surfaces and hands. *J Appl Microbiol*. 2002 Jan;92(3):549–55.

89. Neely A. A Survey of Gram-Negative Bacteria Suvival on Hospital Fabrics and Plastics. *J Burn Care Rehabil*. 2000;21(6):523–7.

90. Hirai Y. Survival of bacteria under dry conditions; from a viewpoint of nosocomial infection. *J Hosp Infect*. 1991 Nov;19(3):191–200.

91. Jawad A, Heritage J, Snelling AM. Influence of relative humidity and suspending menstrua on survival of *Acinetobacter* spp on dry surfaces. *J Clin Microbiol.* 1996;34(12):2881.

92. Eduard W, Dick H. Methods for Quantitative Assessment of Airborne Levels of Noninfectious Microorganisms in Highly Contaminated Work Environments. *Am Ind Hyg J.* 1998;59(2):113–27.

93. Juozaitis A, Willeke K, Grinshpun SA, Donnelly J. Impaction onto a Glass Slide or Agar versus Impingement into a Liquid for the Collection and Recovery of Airborne Microorganisms. *Appl Environ Microbiol*. 1994;60(3):861–70.

94. Griffiths WD, DeCosemo GA. The assessment of bioaerosols: A critical review. *J Aerosol Sci*. 1994;25(8):1425–58.

95. Mukoda TJ, Todd LA, Sobsey MD. PCR and Gene Probes for Detecting Bioaerosols. *J Aerosol Sci.* 1994;25(8):1523–32.

96. Griffiths WD, Stewart IW, Futter SJ, Upton SL, Mark D. The Development of Sampling Methods for the Assessment of Indoor Bioaerosols. *J Aerosol Sci*. 1997;28(3):437–57.

97. Jensen PA, Schafer MP. Sampling and Characterisation of bioaerosols. *NIOSH Man Anal Methods*. 1998;82–112.

98. Li C-S, Hou P-A. Bioaerosol characteristics in hospital clean rooms. *Sci Total Environ.* 2003 Apr 15;305(1-3):169–76.

99. SKC. SKC Biosampler [Internet]. [cited 2011 Aug 23]. Available from: http://www.skcinc.com/instructions/1603.pdf

100. Stetzenbach LD, Buttner MP, Cruz P. Detection and enumeration of airborne biocontaminants. *Curr Opin Biotechnol*. 2004 Jun;15(3):170–4.

101. Peccia J, Hernandez M. Incorporating polymerase chain reaction-based identification, population characterization, and quantification of microorganisms into aerosol science: A review. *Atmos Environ*. 2006 Jul;40(21):3941–61.

102. Verreault D, Rousseau GM, Gendron L, Massé D, Moineau S, Duchaine C. Comparison of Polycarbonate and Polytetrafluoroethylene Filters for Sampling of Airborne Bacteriophages. *Aerosol Sci Technol*. 2010 Feb 10;44(3):197–201.

103. Hospodsky D, Yamamoto N, Peccia J. Accuracy, precision, and method detection limits of quantitative PCR for airborne bacteria and fungi. *Appl Environ Microbiol*. 2010 Nov;76(21):7004–12.

104. Astobiza I, Barandika JF, Ruiz-Fons F, Hurtado A, Povedano I, Juste RA, *et al. Coxiella burnetii* shedding and environmental contamination at lambing in two highly naturally-infected dairy sheep flocks after vaccination. *Res Vet Sci.* 2011 Dec;91(3):e58–63.

105. Hedin G, Rynbäck J, Loré B. New technique to take samples from environmental surfaces using flocked nylon swabs. *J Hosp Infect*. 2010 Aug;75(4):314–7.

106. Galvin S, Dolan A, Cahill O, Daniels S, Humphreys H. Microbial monitoring of the hospital environment: why and how? *J Hosp Infect.* Nov;82(3):143–51.

107. Moore G, Griffith C. Problems associated with traditional hygiene swabbing: the need for in-house standardization. *J Appl Microbiol.* 2007 Oct;103(4):1090–103.

108. Dalmaso G, Bini M, Paroni R, Ferrari M. Qualification of high-recovery, flocked swabs as compared to traditional rayon swabs for microbiological environmental monitoring of surfaces. *J Pharm Sci Technol*. 2008;62(3):191–9.

109. Böcher S, Middendorf B, Westh H, Mellmann A, Becker K, Skov R, *et al*. Semi-selective broth improves screening for methicillin-resistant *Staphylococcus aureus. J Antimicrob Chemother*. 2010 Apr;65(4):717–20.

110. Reisner BS, Shaw S, Huber ME, Carla E, Costa S, Falk PS, *et al*. Comparison of Three Methods to Recover Vancomycin-Resistant Enterococci (VRE) from Perianal and Environmental Samples Collected During a Hospital Outbreak of VRE. *Infect Control Hosp Epidemiol*. 2013;21(12):775–9.

111. Lesmana M, Richie E, Subekti D, Simanjuntak C, Walz SE. Comparison of Direct-Plating and Enrichment Methods for Isolation of *Vibrio cholerae* from Diarrhea Patients. *J Cinical Microbiol.* 1997;35(7):35–8.

112. Platt MW, McLaughlin JC, Gilson GJ, Wellhoner MF, Nims LJ. Increased recovery of group B *Streptococcus* by the inclusion of rectal culturing and enrichment. *Diagn Microbiol Infect Dis.* 1995 Feb;21(2):65–8.

113. Pollock AV, Parida S. The prediction of abdominal surgical infection : the value of an enrichment initial culture of operative parietal wound broth for swabs. *J Hosp Infect.* 1986;8(3):242–7.

114. Zimbro MJ. Manual of Microbiological Culture Media. Maryland: Beckton Dickinson Diagnostics; 2009.

115. Chang C-W, Chou F-C. Methodologies for quantifying culturable, viable, and total *Legionella pneumophila* in indoor air. *Indoor Air*. 2010 Aug;21(4):291–9.

116. Verreault D, Rousseau GM, Gendron L, Massé D, Moineau S, Duchaine C. Comparison of Polycarbonate and Polytetrafluoroethylene Filters for Sampling of Airborne Bacteriophages. *Aerosol Sci Technol*. 2010 Feb 10;44(3):197–201.

117. Coccia AM, Margherita P, Gucci B, Lacchetti I, Paradiso R, Scaini F. Airborne microorganisms associated with waste management and recovery : biomonitoring methodologies. *Ann Ist Super Sanita*. 2010;46(3):288–92.

118. Chen P-S, Li C-S. Sampling Performance for Bioaerosols by Flow Cytometry with Fluorochrome. *Aerosol Sci Technol.* 2005 Mar;39(3):231–7.

119. Oliver JD. Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiol Rev.* 2010 Jul;34(4):415–25.

120. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating. *Proc Natl Acad Sci USA.* 1977;74(12):5463–7.

121. Flores GE, Henley JB, Fierer N. A direct PCR approach to accelerate analyses of human-associated microbial communities. *PLoS One.* 2012 Jan;7(9):e44563.

122. Miller DN, Bryant JE, Madsen EL, Ghiorse WC. Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. *Appl Environ Microbiol.* 1999 Nov;65(11):4715–24.

123. Schrader C, Schielke A, Ellerbroek L, Johne R. PCR inhibitors - occurrence, properties and removal. *J Appl Microbiol.* 2012 Nov;113(5):1014–26.

124. Griffiths RI, Whiteley AS, Anthony G, Donnell O, Bailey MJ, Donnell AGO. Rapid Method for Coextraction of DNA and RNA from Natural Environments for Analysis of Ribosomal DNA- and rRNA-Based Microbial Community Composition. *Appl Environ Microbiol*. 2000;66(12):1–5.

125. Freeman J, Ward E, Calderon C, Mccartney A. A polymerase chain reaction (PCR) assay for the detection of inoculum of *Sclerotinia sclerotiorum. Eur J Plant Pathol.* 2002;108(9):877–86.

126. Kärkkäinen PM, Valkonen M, Hyvärinen A, Nevalainen A, Rintala H. Determination of bacterial load in house dust using qPCR, chemical markers and culture. *J Environ Monit.* 2010 Mar;12(3):759–68.

127. Martin KJ, Rygiewicz PT. Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. *BMC Microbiol.* 2005 Jan;5:28.

128. Haffar M, Gilbride KA. The utility and application of real-time PCR and FISH in the detection of single-copy gene targets in *Escherichia coli* O157 : H7 and *Salmonella typhimurium. Can J Microbiol.* 2010;262(56):254–62.

129. Vadrot C, Bex V, Mouilleseaux A, Squinazi F, Darbord J-C. Detection of *Mycobacterium tuberculosis* complex by PCR in hospital air samples. *J Hosp Infect.* 2004 Dec;58(4):262–7.

130. Rachid CTCC, Santos AL, Piccolo MC, Balieiro FC, Coutinho HLC, Peixoto RS, *et al*. Effect of sugarcane burning or green harvest methods on the Brazilian Cerrado soil bacterial community structure. *PLoS One*. 2013 Jan;8(3):e59342.

131. Liu C., Hungate B., Tobian AA., Serwadda D, Ravel J, Lester R, *et al*. Male Circumcision Significantly Reduces Prevalence and Load of Genital Anaerobic Bacteria. *MBio*. 2013;4(2):1–9.

132. Zoetendal EG, Ben-Amor K, Akkermans AD, Abee T, de Vos WM. DNA isolation protocols affect the detection limit of PCR approaches of bacteria in samples from the human gastrointestinal tract. *Syst Appl Microbiol.* 2001 Nov;24(3):405–10.

133. Wu Y, Shen F, Yao M. Use of gelatin filter and BioSampler in detecting airborne H5N1 nucleotides, bacteria and allergens. *J Aerosol Sci.* 2010 Sep;41(9):869–79.

134. Stahl DA, Lane DJ, Olsen GJ, Pace NR. Analysis of Hydrothermal Vent--Associated Symbionts by Ribosomal RNA Sequences. *Science.* 1984;224(4647):409–11.

135. Brosius J, Palmer ML, Kennedy PJ, Noller HF. Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli. Proc Natl Acad Sci USA*. 1978 Oct;75(10):4801–5.

136. Greengenes [Internet]. [cited 2013 Sep 28]. Available from: http://greengenes.lbl.gov/cgi-bin/JD_Tutorial/nph-16S.cgi

137. Farrelly V, Rainey FA, Stackebrandt E. Effect of genome size and rrn gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. *Appl Environ Microbiol.* 1995 Jul;61(7):2798–801.

138. Chakravorty S, Helb D, Burday M, Connell N, Alland D. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *J Microbiol Methods*. 2007 May;69(2):330–9.

139. Lane DJ, Pace B, Olsen GJ, Stahl D a, Sogin ML, Pace NR. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc Natl Acad Sci USA*. 1985 Oct;82(20):6955–9.

140. Maiwald M, Relman D. Whipple's disease and *Tropheryma whippelii*: secrets slowly revealed. *Clin Infect Dis.* 2001 Feb 1;32(3):457–63.

141. Harris KA, Hartley JC. Development of broad-range 16S rDNA PCR for use in the routine diagnostic clinical microbiology service. *J Med Microbiol.* 2003 Aug 1;52(8):685–91.

142. Yuan I, Xu J, Rooney PJ, Alexander HD, Moore JE. Molecular identification of environmental bacteria in indoor air in the domestic home : Description of a new species of *Exiguobacterium. Int J Environ Health Res.* 2007;17(1):37–41.

143. Heuer H, Krsek M, Baker P, Smalla K. Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Appl Environ Microbiol*. 1997;63(8):3233–41.

144. Huber JA, David B, Welch M, Morrison HG, Huse SM, Neal PR, *et al*. Microbial population structures in the deep marine biosphere. *Science*. 2007 Oct 5;318(5847):97–100.

145. Caporaso JG, Lauber CL, Walters WA, Berg-lyons D, Lozupone CA, Turnbaugh PJ, *et al*. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci USA*. 2011;108(Suppl. 1):4516 – 4522.

146. Spratt DA, Weightman AJ, Wade WG. Diversity of oral asaccharolytic *Eubacterium* species in periodontitis--identification of novel phylotypes representing uncultivated taxa. *Oral Microbiol Immunol*. 1999 Feb;14(1):56–9.

147. Wang Y, Qian P-Y. Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. PLoS One. 2009 Jan;4(10):e7401.

148. Aho EL, Murphy GL, Cannon JG. Distribution of Specific DNA Sequences among Pathogenic and Commensal *Neisseria* Species. *Infect Immun*. 1987;55(4):1009.

149. Tinsley CR, Nassif X. Analysis of the genetic differences between *Neisseria meningitidis* and *Neisseria gonorrhoeae*: two closely related bacteria expressing two different pathogenicities. *Proc Natl Acad Sci USA*. 1996 Oct 1;93(20):11109–14.

150. Perrin A, Bonacorsi S, Carbonnelle E, Talibi D, Dessen P, Nassif X, *et al*. Comparative Genomics Identifies the Genetic Islands That Distinguish *Neisseria meningitidis*, the Agent of Cerebrospinal Meningitis, from Other *Neisseria* Species. *Infect Immun*. 2002;70(12):7063–72.

151. Fox K, Fox A, Rose J, Walla M. Speciation of coagulase negative staphylococci, isolated from indoor air, using SDS PAGE gel bands of expressed proteins followed by MALDI TOF MS and MALDI TOF-TOF MS-MS analysis of tryptic peptides. *J Microbiol Methods.* 2011 Mar;84(2):243–50.

152. Corless CE, Guiver M, Borrow R, Fox AJ. Contamination and Sensitivity Issues with a Real-Time Universal 16S rRNA PCR. *J Clin Microbiol.* 2000;38(5):1747.

153. Wu Z, Wang X-R, Blomquist G. Evaluation of PCR primers and PCR conditions for specific detection of common airborne fungi. *J Environ Monit.* 2002 May 24;4(3):377–82.

154. Hilali F, Saulnier P, Chachaty E, Andremont A. Decontamination of polymerase chain reaction reagents for detection of low concentrations of 16S rRNA genes. *Mol Biotechnol.* 1997 Jun;7(3):207–16.

155. Hugenholtz P, Huber T. Chimeric 16S rDNA sequences of diverse origin are accumulating in the public databases. *Int J Syst Evol Microbiol*. 2003 Jan 1;53(1):289–93.

156. Roth SB, Jalava J, Ruuskanen O, Ruohola A, Nikkari S. Use of an Oligonucleotide Array for Laboratory Diagnosis of Bacteria Responsible for Acute Upper Respiratory Infections. *J Clin Microbiol*. 2004;42(9):4268–74.

157. Hou X-L, Cao Q-Y, Jia H-Y, Chen Z. Pyrosequencing analysis of the gyrB gene to differentiate bacteria responsible for diarrheal diseases. *Eur J Clin Microbiol Infect Dis.* 2008 Jul;27(7):587–96.

158. Lay JO. MALDI-TOF mass spectrometry and bacterial taxonomy. *Trends Anal Chem.* 2000;19(8):507–16.

159. Mkrtchyan H V, Russell CA, Wang N, Cutler RR. Could public restrooms be an environment for bacterial resistomes? *PLoS On*e. 2013 Jan;8(1):e54223.

160. Davies J, Davies D. Origins and evolution of antibiotic resistance. *Microbio*l *Mol Biol Rev*. 2010 Sep;74(3):417–33.

161. Cohen ML. Changing patterns of infectious disease. *Nature.* 2000;406(August):762–7.

162. Vickery K, Deva A, Jacombs A, Allan J, Valente P, Gosbell IB. Presence of biofilm containing viable multiresistant organisms despite terminal cleaning on clinical surfaces in an intensive care unit. *J Hosp Infect*. 2012 Jan;80(1):52–5.

163. Mulvey D, Redding P, Robertson C, Woodall C, Kingsmore P, Bedwell D, *et al.* Finding a benchmark for monitoring hospital cleanliness. *J Hosp Infect*. 2011 Jan;77(1):25–30.

164. White LF, Dancer SJ, Robertson C, McDonald J. Are hygiene standards useful in assessing infection risk? *Am J Infect Control.* 2008 Jun;36(5):381–4.

165. NICE. Prevention and control of healthcare-associated infections. 2011.

166. Curtis C, Shetty N. Recent trends and prevention of infection in the neonatal intensive care unit. *Curr Opin Infect Dis*. 2008 Aug;21(4):350–6.

167. Posfay-Barbe K.M, Zerr D.M PD. Infection control in paediatrics. *Lancet* Infect Dis. 2008 Jun;8:19–31.

168. Utsumi M, Makimoto K, Quroshi N, Ashida N. Types of infectious outbreaks and their impact in elderly care facilities: a review of the literature. *Age Ageing.* 2010 May;39(3):299–305.

169. Tambyah PA OJ. Catheter-associated urinary tract infection. *Curr Opin Infect Dis.* 2012;25(4):365–70.

170. Panagea S, Winstanley C, Walshaw MJ, Ledson MJ, Hart CA. Environmental contamination with an epidemic strain of *Pseudomonas aeruginosa* in a Liverpool cystic fibrosis centre, and study of its survival on dry surfaces. *J Hosp Infect.* 2005 Feb;59(2):102–7.

171. Morse A. Reducing Healthcare Associated Infections in Hospitals in England. London; 2009.

172. Trajman A, Menzies D. Occupational respiratory infections. *Curr Opin Pulm Med.* 2010 May;16(3):226–34.

173. Chen P-S, Li C-S. Concentration Profiles of Airborne *Mycobacterium tuberculosis* in a Hospital. *Aerosol Sci Technol.* 2008 Jan 24;42(3):194–200.

174. Best EL, Fawley WN, Parnell P, Wilcox MH. The potential for airborne dispersal of *Clostridium difficile* from symptomatic patients. *Clin Infect Dis*. 2010 Jun 1;50(11):1450–7.

175. Barbolla RE, Centrón D, Maimone S, Rospide F, Salgueira C, Altclas J, *et al*. Molecular epidemiology of *Acinetobacter baumannii* spread in an adult intensive care unit under an endemic setting. *Am J Infect Control.* 2008 Aug;36(6):444–52.

176. Smith SJ, Young V, Robertson C, Dancer SJ. Where do hands go? An audit of sequential hand-touch events on a hospital ward. *J Hosp Infect.* 2012 Mar;80(3):206–11.

177. Wilson APR, Smyth D, Moore G, Singleton J, Jackson R, Gant V, *et al*. The impact of enhanced cleaning within the intensive care unit on contamination of the near-patient environment with hospital pathogens: a randomized crossover study in critical care units in two hospitals. *Crit Care Med*. 2011 Apr;39(4):651–8.

178. Bodey GP, Bolivar R, Fainstein V, Jadeja L. Infections Caused by *Pseudomonas aeruginosa. Rev Infect Dis.* 2013;5(2):279–313.

179. Bearden A, Friedrich TC, Goldberg TL, Byrne B, Spiegel C, Schult P, *et al.* An outbreak of the 2009 influenza a (H1N1) virus in a children's hospital. *Influenza Other Respi Viruses*. 2012 Sep;6(5):374–9.

180. Sun L, Klein EY, Laxminarayan R. Seasonality and Temporal Correlation between Community Antibiotic Use and Resistance in the United States. *Clin Infect Dis.* 2012;55(5):687–94.

181. Diene SM, Rolain J-M. Investigation of antibiotic resistance in the genomic era of multidrug-resistant Gram-negative bacilli, especially Enterobacteriaceae, *Pseudomonas* and *Acinetobacter. Expert Rev Anti Infect Ther.* 2013 Mar;11(3):277–96.

182. Khan A, Dancer S, Humphreys H. Priorities in the prevention and control of multidrug-resistant Enterobacteriaceae in hospitals. *J Hosp Infect.* 2012;82(2):85–93.

183. Lau SH, Kaufmann ME, Livermore DM, Woodford N, Willshaw GA, Cheasty T, *et al*. UK epidemic *Escherichia coli* strains A-E, with CTX-M-15 beta-lactamase, all belong to the international O25:H4-ST131 clone. *J Antimicrob Chemother.* 2008 Dec;62(6):1241–4.

184. Weber DJ, Rutala WA. Understanding and Preventing Transmission of Healthcare-Associated Pathogens Due to the Contaminated Hospital Environment. *Infect Control Hosp Epidemiol.* 2013 May;34(5):449–52.

185. Sammons JS, Toltzis P, Zaoutis TE. *Clostridium difficile* Infection in children. *JAMA Pediatr*. 2013 Jun;167(6):567–73.

186. Baron S. Medical Microbiology. 4th ed. Galveston, editor. Texas; 1996.

187. Rathinavelu S, Zavros Y, Merchant JL. *Acinetobacter lwoffii* infection and gastritis. *Microbes Infect.* 2003;5:651–7.

188. Pellegrino FLPC, Vieira V V, Baio PVP, dos Santos RMR, dos Santos ALA, Santos NGDB, *et al. Acinetobacter soli* as a cause of bloodstream infection in a neonatal intensive care unit. *J Clin Microbiol.* 2011 Jun;49(6):2283–5.

189. Tayabali AF, Nguyen KC, Shwed PS, Crosthwait J, Coleman G, Seligy VL. Comparison of the virulence potential of *Acinetobacter* strains from clinical and environmental sources. *PLoS One.* 2012 Jan;7(5):e37024.

190. Hu J, Robinson JL. Systematic review of invasive *Acinetobacter* infections in children. *Can J Infect Dis Med Microbiol.* 2010 Jan;21(2):83–8.

191. Thom KA, Johnson JK, Lee MS, Harris AD. Environmental contamination because of multidrug-resistant *Acinetobacter baumannii* surrounding colonized or infected patients. *Am J Infect Control*. 2011 Nov;39(9):711–5.

192. Spellberg B, Bonomo RA. "Airborne assault": a new dimension in *Acinetobacter baumannii* transmission. *Crit Care Med.* 2013 Aug;41(8):2042–4.

193. Gordon NC, Wareham DW. Multidrug-resistant *Acinetobacter baumannii:* mechanisms of virulence and resistance. *Int J Antimicrob Agents*. 2010 Mar;35(3):219–26.

194. Munoz-Price LS, Fajardo-Aquino Y, Arheart KL, Cleary T, DePascale D, Pizano L, *et al.* Aerosolization of *Acinetobacter baumannii* in a trauma ICU. *Crit Care Med*. 2013 Aug;41(8):1915–8.

195. Kim SE, Park S-H, Park HB, Park K-H, Kim S-H, Jung S-I, *et al*. Nosocomial *Pseudomonas putida* Bacteremia: High Rates of Carbapenem Resistance and Mortality. *Chonnam Med J.* 2012 Aug;48(2):91–5.

196. Oğuz SS, Unlü S, Saygan S, Dilli D, Erdoğan B, Dilmen U. Rapid control of an outbreak of *Pseudomonas putida* in a tertiary neonatal intensive care unit. *J Hosp Infect.* 2010 Dec;76(4):361–2.

197. Grimaldi D, Podglajen I, Aubert A, Buu-Hoï A, Diebold B, Mainardi J-L. Case of indolent endocarditis due to *Pseudomonas stutzeri* with genetic evidence of relapse after 4 years. *J Clin Microbiol*. 2009 Feb;47(2):503–4.

198. Pendleton JN, Gorman SP, Gilmore BF. Clinical relevance of the ESKAPE pathogens. *Expert Rev Anti Infect Ther.* 2013 Mar;11(3):297–308.

199. Cattoir V, Leclercq R. Twenty-five years of shared life with vancomycin-resistant enterococci: is it time to divorce? *J Antimicrob Chemother*. 2013 Apr;68(4):731–42.

200. Hollenbeck BL, Rice LB. Intrinsic and acquired resistance mechanisms in enterococcus. *Virulence*. 2012 Aug 15;3(5):421–33.

201. Haugland RA, Brinkman N, Vesper SJ. Evaluation of rapid DNA extraction methods for the quantitative detection of fungi using real-time PCR analysis. *J Microbiol Methods.* 2002 Aug;50(3):319–23.

202. Bellanger A-P, Reboux G, Murat J-B, Bex V, Millon L. Detection of *Aspergillus fumigatus* by quantitative polymerase chain reaction in air samples impacted on low-melt agar. *Am J Infect Control.* 2010 Apr;38(3):195–8.

203. Flannigan B. Air sampling for fungi in indoor environments. *J Aerosol Sci.* 1997;28(3):381–92.

204. Reponen T, Singh U, Schaffer C, Vesper S, Johansson E, Adhikari A, *et al*. Visually observed mold and moldy odor versus quantitatively measured microbial exposure in homes. *Sci Total Environ.* 2010 Oct 15;408(22):5565–74.

205. Dancer SJ. Hospital cleaning in the 21st century. *Eur J Clin Microbiol Infect Dis.* 2011 Dec;30(12):1473–81.

206. Foster CB, Sabella C. Health care--associated infections in children. *JAMA.* 2011 Apr 13;305(14):1480–1.

207. Tregoning JS, Schwarze J. Respiratory viral infections in infants: causes, clinical symptoms, virology, and immunology. *Clin Microbiol Rev.* 2010 Jan;23(1):74–98.

208. Martin ET, Fairchok MP, Kuypers J, Magaret A, Zerr DM, Wald A, *et al.* Frequent and prolonged shedding of bocavirus in young children attending daycare. *J Infect Dis*. 2010 Jun 1;201(11):1625–32.

209. Murata T, Katsushima N, Mizuta K, Muraki Y, Hongo S, Matsuzaki Y. Prolonged norovirus shedding in infants <or=6 months of age with gastroenteritis. *Pediatr Infect Dis J*. 2007 Jan;26(1):46–9.

210. Najioullah F, Tissot Guerraz F, Thouvenot D, Milon M-P, Lachaux A, Floret D. Nosocomial infections due to adenovirus in a paediatric unit. *Pathol Bio*l. 2004 Feb;52(1):16–20.

211. Adler SP. Molecular epidemiology of cytomegalovirus: a study of factors affecting transmission among children at three day-care centres. *Pediatr Infect Dis J.* 1991;10(8):584–90.

212. Miles A., Misra S., Irwin J. The Estimation of the Bactericidal Power of the Blood. *J Hyg*. 1938;38(6):732–49.

213. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol.* 1991 Jan;173(2):697–703.

214. Bonder MJ, Abeln S, Zaura E, Brandt BW. Comparing clustering and pre-processing in taxonomy analysis. Bioinformatics. 2012 Nov 15;28(22):2891–7.

215. Life Technologies. Top Ten Most Common Real-Time qRT-PCR Pitfalls [Internet]. [cited 2013 Aug 19]. Available from: http://www.invitrogen.com/site/us/en/home/References/Ambion-Tech-Support/rtpcr-analysis/general-articles/ten-most-common-real-time-qrt-pcr-pitfalls.html

216. Drancourt M, Bollet C, Carlioz A, Martelin R. 16S Ribosomal DNA Sequence Analysis of a Large Collection of Environmental and Clinical Unidentifiable Bacterial Isolates. *J Clin Microbiol.* 2000;30(10):3623–30.

217. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, *et al*. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods.* 2011;7(5):335–6.

218. Edgar RC. USEARCH Software and Documentation [Internet]. 2013 [cited 2013 Sep 27]. Available from: http://drive5.com/usearch/

219. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, *et al*. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol.* 2006 Jul;72(7):5069–72.

220. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol.* 2007 Aug;73(16):5261–7.

221. Bokulich NA, Subramanian S, Faith JJ, Gevers D, Gordon JI, Knight R, *et al.* Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat Methods*. 2013;10(1):57–60.

222. Baker GC, Smith JJ, Cowan DA. Review and re-analysis of domain-specific 16S primers. *J Microbiol Methods.* 2003 Dec;55(3):541–55.

223. Hong S, Bunge J, Leslin C, Jeon S, Epstein S. Polymerase chain reaction primers miss half of rRNA microbial diversity. *ISME J.* 2009;3:1365–73.

224. Niskanen A, Pohja MS. Comparative studies on the sampling and investigation of microbial contamination of surfaces by the contact plate and swab methods. *J Appl Microbiol.* 1977;42(1):53 – 63.

225. Qiagen [Internet]. QIAamp DNA Mini Kit. [cited 2013 Sep 29]. Available from: http://www.qiagen.com/products/catalog/sample-technologies/dna-sample-technologies/genomic-dna/qiaamp-dna-mini-kit#productdetails

226. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, *et al*. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J*. 2012 Aug;6(8):1621–4.

227. Ahn J-H, Kim B-Y, Song J, Weon H-Y. Effects of PCR cycle number and DNA polymerase type on the 16S rRNA gene pyrosequencing analysis of bacterial communities. *J Microbiol.* 2012 Dec;50(6):1071–4.

228. Huggett JF, Novak T, Garson JA, Green C, Morris-Jones SD, Miller RF, *et al.* Differential susceptibility of PCR reactions to inhibitors: an important and unrecognised phenomenon. *BMC Res Notes.* 2008 Jan;1:70.

229. Butler J, Hill C. Scientific Issues with Analysis of Low Amounts of DNA. Promega Corporation Web site [Internet]. 2010 [cited 2013 Sep 28]. p. 1–9. Available from: http://www.promega.co.uk/resources/articles/profiles-in-dna/2010/scientific-issues-with-analysis-of-low-amounts-of-dna/

230. Chandler DP, Fredrickson JK, Brockman FJ. Effect of PCR template concentration on the composition and distribution of total community 16S rDNA clone libraries. *Mol Ecol.* 1997 May;6(5):475–82.

231. HPA. A Children's Environment and Health Strategy for the UK. London; 2009.

232. Cauchemez S, Ferguson NM, Wachtel C, Tegnell A, Saour G, Duncan B, *et al.* Closure of schools during an influenza pandemic. *Lancet Infect Dis.* 2009 Aug;9(8):473–81.

233. Salathé M, Kazandjieva M, Woo J, Levis P, Feldman MW, Jones JH. A high-resolution human contact network for infectious disease transmission. *Proc Natl Acad Sci USA.* 2010;107(51):22020–5.

234. Bayliss J. Children and young people around the UK. *Reg Trends*. 2009;41:2–30.

235. Pienaar E, Fluitt AM, Whitney SE, Freifeld AG, Viljoen HJ. A model of tuberculosis transmission and intervention strategies in an urban residential area. *Comput Biol Chem*. 2010 May;34(2):86–96.

236. Edelson PJ, Phypers M. TB transmission on public transportation: a review of published studies and recommendations for contact tracing. *Travel Med Infect Dis.* 2011 Jan;9(1):27–31.

237. Alzubaidi H, Carr J, Councell R, Johnson G. Households Below Average Income. An analysis of the income distribution 1994/95 - 2011/12. 2013.

238. Anonymous. Travel in London. 2010.

239. Gushulak BD, MacPherson DW. Globalization of infectious diseases: the impact of migration. *Clin Infect Dis.* 2004 Jun 15;38(12):1742–8.

240. Barnett ED, Walker PF. Role of immigrants and migrants in emerging infectious diseases. *Med Clin North Am*. 2008 Nov;92(6):1447–58

241. HaCIRIC. Users' behaviour within bed spaces on the Paediatric Intensive Care Unit (PICU) Health and Care Infrastructure Research and Innovation Centre and Great Ormond Street Hospital Executive Summary Report Draft 01. 2011 p. 1–6.

242. Gravesen S, Larsen L, Gyntelberg F, Skov P. Demonstration of Microorganisms and Dust in Schools and Offices. *Allergy.* 1986 Sep;41(7):520–5.

243. WHO. Five Moments for Hand Hygiene [Internet]. Clean Care is Safer Care. 2013 [cited 2013 Dec 1]. Available from: http://www.who.int/gpsc/tools/Five_moments/en/

244. Frange P, Touzot F, Debré M, Héritier S, Leruez-Ville M, Cros G, *et al.* Prevalence and clinical impact of norovirus fecal shedding in children with inherited immune deficiencies. *J Infect Dis.* 2012 Oct;206(8):1269–74.

245. Norovirus Working Party. Guidelines for the management of norovirus outbreaks in acute and community health and social care settings. 2011.

246. Kundu S, Lockwood J, Depledge DP, Chaudhry Y, Aston A, Rao K, et al. Next-Generation Whole Genome Sequencing Identifies the Direction of Norovirus Transmission in Linked Patients. *Clin Infect Dis.* 2013 May 28;1–8.

247. Rennick JE, Johnston CC, Dougherty G, Platt R, Ritchie JA. Children's Psychological Responses After Critical Illness and Exposure to Invasive Technology. *J Dev Behav Pediatr.* 2002 Jun;23(3):133–44.

248. Resch B, Michel-Behnke I. Respiratory syncytial virus infections in infants and children with congenital heart disease: update on the evidence of prevention with palivizumab. *Curr Opin Cardiol.* 2013 Jan;28(2):85–91.

249. Straub TM, Höner zu Bentrup K, Orosz-Coghlan P, Dohnalkova A, Mayer BK, Bartholomew RA, *et al*. In vitro cell culture infectivity assay for human noroviruses. *Emerg Infect Dis*. 2007 Mar;13(3):396–403.

250. Patel MM, Hall AJ, Vinjé J, Parashar UD. Noroviruses: a comprehensive review. *J Clin Virol*. 2009 Jan;44(1):1–8.

251. Wu HM, Fornek M, Schwab KJ, Amy R, Gibson K, Schwab E, *et al.* A Norovirus Outbreak at a Long Term-Care Facility: The Role of Environmental Surface Contamination. *Infect Control Hosp Epidemiol.* 2005;26(10):802–10.

252. Piedra P. Adenovirus in the neonatal intensive care unit: formidable, forgotten foe. *J Pediatr*. 2005;146:447–8.

253. Tebruegge M, Curtis N. Adenovirus: an overview for pediatric infectious diseases specialists. *Pediatr Infect Dis J.* 2012 Jun;31(6):626–7.

254. Hale GA, Heslop HE, Krance RA, Brenner MA, Jayawardene D, Srivastava DK, *et al*. Adenovirus infection after pediatric bone marrow transplantation. *Bone Marrow Transplant.* 1999 Feb;23(3):277–82.

255. Peled N. Adenovirus Infection in Hospitalized Immunocompetent Children. *Clin Pediatr*. 2004 Apr 1;43(3):223–9.

256. Echavarría M. Adenoviruses in immunocompromised hosts. *Clin Microbiol Rev.* 2008 Oct;21(4):704–15.

257. Jalal H, Bibby DF, Tang JW, Bennett J, Kyriakou C, Peggs K, *et al.* First Reported Outbreak of Diarrhea Due to Adenovirus Infection in a Hematology Unit for Adults. *J Clin Microbiol.* 2005;43(6):2575–80.

258. Chaberny IE, Schnitzler P, Geiss HK, Wendt C. An outbreak of epidemic keratoconjunctivtis in a pediatric unit due to adenovirus type 8. *Infect Control Hosp Epidemiol.* 2003 Jul;24(7):514–9.

259. Wan G-H, Huang C-G, Huang Y-C, Huang J-P, Yang S-L, Lin T-Y, *et al*. Surveillance of airborne adenovirus and *Mycoplasma pneumoniae* in a hospital pediatric department. *PLoS One. 2*012 Jan;7(3):e33974.

260. Baek YH, Choi EH, Song M-S, Pascua PNQ, Kwon H, Park S-J, *et al*. Prevalence and genetic characterization of respiratory syncytial virus (RSV) in hospitalized children in Korea. *Arch Virol.* 2012 Jun;157(6):1039–50.

261. Nair H, Verma VR, Theodoratou E, Zgaga L, Huda T, Simões EAF, *et al*. An evaluation of the emerging interventions against Respiratory Syncytial Virus (RSV)-associated acute lower respiratory infections in children. *BMC Public Health.* 2011 Jan;11(Suppl 3):S30.

262. Noyola DE, Mandeville PB. Effect of climatological factors on respiratory syncytial virus epidemics. *Epidemiol Infect*. 2008 Oct;136(10):1328–32.

263. Woods CR, Bryant KA. Viral Infections in Children with Community-Acquired Pneumonia. *Curr Infect Dis Rep.* 2013 Feb 1;

264. Bont L. Nosocomial RSV infection control and outbreak management. *Paediatr Respir Rev.* 2009 Jun;10 Suppl 1:16–7.

265. Rechsteiner J, Winkler KC. Inactivation of Respiratory Syncytial Virus in Aerosol. *J Gen Virol.* 1969 Oct 1;5(3):405–10.

266. Yusuf S, Piedimonte G, Auais A, Krishnan S, Van Caesseele P. The relationship of meteorological conditions to the epidemic activity of respiratory syncytial virus. *Epidemiol Infect.* 2007 Oct;135(7):1077–90.

267. HPA. Laboratroy Reports of RSV by age, by date of specimen, 2012/13 [Internet]. 2013 [cited 2013 Jul 20]. Available from: http://www.hpa.org.uk/web/HPAweb&HPAwebStandard/HPAweb_C/1223971255821

268. Hervás D, Reina J, Hervás JA. Meteorologic conditions and respiratory syncytial virus activity. *Pediatr Infect Dis J.* 2012 Oct;31(10):e176–81.

269. Van den Hoogen, BG, De Jong JC, Groen J, Kuiken T, De Groot R, Fouchier RAM, Osterhaus ADM. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. *Nat Med.* 2001;7:719–24.

270. Posfay-Barbe KM. Infections in pediatrics: old and new diseases. *Swiss Med Wkly.* 2012 Jan;142:13654.

271. Jewell D. Human metapneumovirus. *Br J Gen Pract.* 2005;(February):84–5.

272. Jartti T, Jartti L, Ruuskanen O, Söderlund-Venermo M. New respiratory viral infections. *Curr Opin Pulm Med*. 2012 May;18(3):271–8.

273. Peiris JSM, Tang W-H, Chan K-H, Khong P-L, Guan Y, Lau Y-L, *et al.* Children with respiratory disease associated with metapneumovirus in Hong Kong. *Emerg Infect Dis.* 2003 Jun;9(6):628–33.

274. Debur MC, Vidal LR, Stroparo E, Nogueira MB, Almeida SM, Takahashi GA, *et al*. Human metapneumovirus infection in hematopoietic stem cell transplant recipients. *Transpl Infect Dis.* 2010 Apr;12(2):173–9.

275. Patton JT. Rotavirus Diversity and Evolution in the Post-Vaccine World. *Discov Med.* 2012;13(68):85–97.

276. Yen C, Tate J, Patel MM, Cortese MM, Lopman B, Fleming J, *et a*l. Update on global impact and future priorities Rotavirus vaccines. *Hum Vaccin.* 2011;7(12):1282–90.

277. Dennehy PH. Rotavirus Infection. An Update on Management and Prevention. *Adv Pediatr.* 2012;59(1):47–74.

278. Fischer TK, Bresee JS, Glass RI. Rotavirus vaccines and the prevention of hospital-acquired diarrhea in children. *Vaccine*. 2004;22S:S49–S54.

279. Dennehy Penelope H, Nelson SM, Crowley BA, Saracen C. Detection of rotavirus RNA in hospital air samples by Polymerase Chain Reaction (PCR). *Pediatr Res*. 1998;43(4))S2.

280. Abad FX, Pintó RM, Bosch A. Survival of enteric viruses on environmental fomites. *Appl Environ Microbiol.* 1994 Oct;60(10):3704–10.

281. Bruijning-Verhagen P, Quach C, Bonten M. Nosocomial rotavirus infections: a meta-analysis. *Pediatrics*. 2012 Apr;129(4):e1011–9.

282. Carr S. Seasonal and pandemic influenza: an overview with pediatric focus. *Adv Pediatr.* 2012 Jan;59(1):75–93.

283. Enstone JE, Myles PR, Openshaw PJM, Gadd EM, Lim WS, Semple MG, *et al.* Nosocomial pandemic (H1N1) 2009, United Kingdom, 2009-2010. *Emerg Infect Dis.* 2011 Apr;17(4):592–8.

284. Khandaker G, Rashid H, Zurynski Y, Richmond PC, Buttery J, Marshall H, *et al.* Nosocomial vs community-acquired pandemic influenza A (H1N1) 2009: a nested case-control study. *J Hosp Infect*. 2012 Oct;82(2):94–100.

285. Jones RM. Critical review and uncertainty analysis of factors influencing influenza transmission. *Risk Anal.* 2011 Aug;31(8):1226–42.

286. Blachere FM, Lindsley WG, Pearce TA, Anderson SE, Fisher M, Khakoo R, *et al.* Measurement of airborne influenza virus in a hospital emergency department. *Clin Infect Dis.* 2009 Feb 15;48(4):438–40.

287. Yang W, Marr LC. Dynamics of airborne influenza A viruses indoors and dependence on humidity. *PLoS One.* 2011 Jan;6(6):e21481.

288. Belser JA, Maines TR, Tumpey TM, Katz JM. Influenza A virus transmission: contributing factors and clinical implications. *Expert Rev Mol Med.* 2010 Jan;12(December):e39.

289. Ludwig A, Hengel H. Epidemiological Impact and Disease Burden of Congenital Cytomegalovirus Infection in Europe. *Eurosurveillance.* 2009;14(9):1–7.

290. HPA. Factsheet on Cytomegalovirus [Internet]. 2012 [cited 2013 Jul 20]. Available from: http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1194947324100

291. Grilli E, Galati V, Bordi L, Taglietti F, Petrosillo N. Cytomegalovirus pneumonia in immunocompetent host: case report and literature review. *J Clin Virol.* 2012 Dec;55(4):356–9.

292. Eddleston M, Peacock S, Juniper M, Warrell DA. Severe cytomegalovirus infection in immunocompetent patients. *Clin Infect Dis.* 1997 Jan;24(1):52–6.

293. Duerkop BA, Hooper LV. Resident viruses and their interactions with the immune system. *Nat Immunol*. 2013 Jun 18;14(7):654–9.

294. Delwart E. A roadmap to the human virome. *PLoS Pathog*. 2013 Feb;9(2):e1003146.

295. Roux S, Enault F, Robin A, Ravet V, Personnic S, Theil S, *et al.* Assessing the diversity and specificity of two freshwater viral communities through metagenomics. *PLoS One.* 2012 Jan;7(3):e33641.

296. Hurwitz BL, Sullivan MB. The Pacific Ocean virome (POV): a marine viral metagenomic dataset and associated protein clusters for quantitative viral ecology. *PLoS One*. 2013 Jan;8(2):e57355.

297. Tyagi AK, Pradier A, Baumer O, Uppugunduri CRS, Huezo-Diaz P, Posfay-Barbe KM, *et al.* Validation of SYBR Green based quantification assay for the detection of human Torque Teno virus titers from plasma. *Virol J.* 2013 Jan;10:191.

298. Bonroy C, Vankeerberghen A, Boel A, De Beenhouwer H. Use of a multiplex real-time PCR to study the incidence of human metapneumovirus and human respiratory syncytial virus infections during two winter seasons in a Belgian paediatric hospital. *Clin Microbiol Infect*. 2007 May;13(5):504–9.

299. Tseng C, Li C. Concentration of Airborne Viruses in Pediatrics Department Measured using Real-Time qPCR Coupled to an Air-Sampling Filter Method. *Epidemiology*. 2008;19(6):S195.

300. Reponen T. Methodologies for Assessing Bioaerosol Exposures. In: *Encyclopedia of Environmental Health.* Burlington: Elsevier; 2011. p722–30.

301. Stowell JD, Forlin-passoni D, Din E, Radford K, Brown D, White A, *et al.* Cytomegalovirus Survival on Common Environmental Surfaces : Opportunities for Viral Transmission. *J Infect Dis.* 2012;205(15):211–4.

302. Forster JL, Harkin VB, Graham DA, McCullough SJ. The effect of sample type, temperature and RNAlater on the stability of avian influenza virus RNA. *J Virol Methods*. 2008 Apr;149(1):190–4.

303. Heim A, Ebnet C, Harste G, Pring-Akerblom P. Rapid and quantitative detection of human adenovirus DNA by real-time PCR. *J Med Virol.* 2003 Jun;70(2):228–39.

304. Preiser W, Brink NS, Ayliffe U, Peggs KS, Mackinnon S, Tedder RS, *et al*. Development and clinical application of a fully controlled quantitative PCR assay for cell-free cytomegalovirus in human plasma. *J Clin Virol.* 2003 Jan;26(1):49–59.

305. Hu A, Colella M, Tam JS, Rappaport R, Cheng S-M. Quantitation of Respiratory Syncytial Virus A and B by Real-Time PCR Simultaneous Detection and Subgrouping. *J Clin Microbiol.* 2003;41(1):18–24.

306. La Rosa G, Fratini M, Libera S Della, Iaconelli M, Muscillo M. Viral infections acquired indoors through airborne , droplet or contact transmission. *Ann Ist Super Sanita*. 2013;49(2):124–32.

307. Ganime AC, Carvalho-Costa FA, Mendonça MCL, Vieira CB, Santos M, Costa Filho R, *et al.* Group A rotavirus detection on environmental surfaces in a hospital intensive care unit. *Am J Infect Control*. 2012 Aug;40(6):544–7.

308. Couch R, Knight V, Douglas J, Black S, Hamory B. The minimal infectious dose of adenovirus type 4; the case for transmission by viral aerosol. *Trans Am Clin Climatol Assoc.* 1969;80:205–11.

309. Echavarria M, Kolavic SA, Cersovsky S, Mitchell F, Sanchez JL, Polyak C, *et al.* Detection of adenoviruses (AdV) in culture-negative environmental samples by PCR during an AdV-associated respiratory disease outbreak. *J Clin Microbiol.* 2000 Aug;38(8):2982–4.

310. McCluskey R, Sandin R, Greene J. Detection of airborne cytomegalovirus in hospital rooms of immunocompromised patients. *J Virol Methods.* 1996 Jan;56(1):115–8.

311. Griffin JS, Plummer JD, Long SC. Torque teno virus: an improved indicator for viral pathogens in drinking waters. *Virol J.* 2008 Jan;5:112.

312. Chikasue K, Kimura M, Ikeda K, Ohnishi T, Kawanishi S, Iio T, *et al*. Detection of Torque teno virus DNA in exhaled breath by polymerase chain reaction. *Acta Med Okayama*. 2012 Jan;66(5):387–97.

313. Pica A, Bouvier NM. Environmental Factors Affecting the Transmission of Respiratory Viruses. *Curr Opin Virol*. 2013;2(1):90–5.

314. Getchell-White S, Donowitz LG, Groschel DHM. The Inanimate Environment of an Intensive Care Unit as a Potential Source of Nosocomial Bacteria: Evidence for Long Survival of *Acinetobacter calcoaceticus*. *Infect Control Hosp Epidemiol.* 1989;10(9):402–7.

315. Lemmen SW, Häfner H, Zolldann D, Stanzel S, Lütticken R. Distribution of multi-resistant Gram negative versus Gram positive bacteria in the hospital inanimate environment. *J Hosp Infect.* 2004 Mar;56(3):191–7.

316. Kaatz GW, Gitlin SD, Schaberg DR, Wilson KH, Kauffman CA, Seo SM, *et al.* Acquisition of *Clostridium difficile* from the hospital environment. *Am J Epidemiol.* 1988 Jun;127(6):1289–94.

317. Al-Hamad A, Maxwell S. How clean is clean? Proposed methods for hospital cleaning assessment. *J Hosp Infect.* 2008 Dec;70(4):328–34.

318. Dansby W, Purdue G, Hunt J, Arnoldo B, Phillips D, Moody B, *et al*. Aerosolization of methicillin-resistant *Staphylococcus aureus* during an epidemic in a burn intensive care unit. *J Burn Care Res*. 1985;29(2):331–7.

319. Roberts K. Bioaerosol Production on a Respiratory Ward. Indoor *Built Environ*. 2006 Feb 1;15(1):35–40.

320. Cobben N, Drent M, Jonkers M, Wouters EF, Vaneechoutte M, Stobberingh EE. Outbreak of severe *Pseudomonas aeruginosa* respiratory infections due to contaminated nebulizers. *J Hosp Infect.* 1996 May;33(1):63–70.

321. Wilson AP, Hayman S, Whitehouse T, Cepeda J, Kibbler C, Shaw S, *et al*. Importance of the environment for patient acquisition of methicillin-resistant *Staphylococcus aureus* in the intensive care unit: A baseline study. *Crit Care Med*. 2007 Oct;35(10):2275–9.

322. Wilson A, Ostro P, Magnussen M, Cooper B. Laboratory and in-use assessment of methicillin-resistant *Staphylococcus aureus* contamination of ergonomic computer keyboards for ward use. *Am J Infect Control.* 2008 Dec;36(10):e19–25.

323. Bandi S, Uddin L, Milward K, Aliyu S, Makwana N. How clean are our stethoscopes and do we need to clean them? *J Infect.* 2008 Oct;57(4):355–6.

324. Kooken JM, Fox KF, Fox A. Characterization of *Micrococcus* strains isolated from indoor air. *Mol Cell Probes.* 2012 Feb;26(1):1–5.

325. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, *et al*. Initial sequencing and analysis of the human genome. *Nature.* 2001 Feb 15;409(6822):860–921.

326. Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, *et al*. The Sequence of the Human Genome. *Science*. 200 29:1304–51.

327. Nowrousian M. Next-generation sequencing techniques for eukaryotic microorganisms: sequencing-based solutions to biological problems. *Eukaryot Cell. 2*010 Sep;(9):1300–10.

328. Zhang J, Chiodini R, Badr A, Zhang G. The impact of next-generation sequencing on genomics. J Genet Genomics. 2011;38(3):95–109.

329. Loman NJ, Misra RV, Dallman TJ, Constantinidou C, Gharbia SE, Wain J, *et al*. Performance comparison of benchtop high-throughput sequencing platforms. *Nat Biotechnol*. 2012 May;30(5):434–9.

330. Blainey PC, Milla CE, Cornfield DN, Quake SR. Quantitative analysis of the human airway microbial ecology reveals a pervasive signature for cystic fibrosis. *Sci Transl Med.* 2012 Sep 26;4(153):153ra130.

331. Beck JM, Young VB, Huffnagle GB. The microbiome of the lung. *Transl Res.* 2012 Oct;160(4):258–66.

332. Han M, Hhuang Y, LiPuma JJ, Boushey HA, Boucher R, Cookson WO, *et al*. Significance of the microbiome in obstructive lung disease. *Thorax*. 2013;67(5):456–63.

333. Glenn T. Field Guide to Next Generation DNA Sequencers. *Mol Ecol Resour*. 2011;11(5):759–69.

334. Hamady M, Knight R. Microbial community profiling for human microbiome projects: Tools, techniques, and challenges. *Genome Res.* 2009 Jul;19(7):1141–52.

335. Zhu Z, Jenkins G, Zhang W, Zhang M, Guan Z, Yang CJ. Single-molecule emulsion PCR in microfluidic droplets. *Anal Bioanal Chem.* 2012 Jun;403(8):2127–43.

336. Shao K, Ding W, Wang F, Li H, Ma D, Wang H. Emulsion PCR: a high efficient way of PCR amplification of random DNA libraries in aptamer selection. *PLoS One*. 2011 Jan;6(9):e24910.

337. Williams R, Peisajovich SG, Miller OJ, Magdassi S, Tawfik DS, Griffiths AD. Amplification of complex gene libraries by emulsion PCR. *Nat Methods.* 2006;3(7):545–50.

338. Ronaghi M. Pyrosequencing Sheds Light on DNA Sequencing. *Genome Res.* 2001;11:3–11.

339. Tucker T, Marra M, Friedman JM. Massively parallel sequencing: the next big thing in genetic medicine. *Am J Hum Genet*. 2009 Aug;85(2):142–54.

340. Metzker ML. Sequencing technologies - the next generation. *Nat Rev Genet*. 2010;11(1):31–46.

341. Rothberg JM, Hinz W, Rearick TM, Schultz J, Mileski W, Davey M, *et al.* An integrated semiconductor device enabling non-optical genome sequencing. *Nature.* 2011 Jul 21;475(7356):348–52.

342. ABI. Overview of SOLiD^{TM} Sequencing Chemistry [Internet]. [cited 2013 Oct 9]. Available from: http://www.appliedbiosystems.com

343. Eid J, Fehr A, Gray J, Luong K, Lyle J, Otto G, *et al*. Real-time DNA sequencing from single polymerase molecules. *Science*. 2009 Jan 2;323(5910):133–8.

344. Fichot EB, Norman RS. Microbial phylogenetic profiling with the Pacific Biosciences sequencing platform. *Microbiome.* 2013;1(1):10.

345. Quail MA, Smith M, Coupland P, Otto TD, Harris SR, Connor TR, *et al.* A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. *BMC Genomics*. 2012 Jan;13(1):341.

346. Pinto AJ, Raskin L. PCR biases distort bacterial and archaeal community structure in pyrosequencing datasets. *PLoS One.* 2012 Jan;7(8):e43093.

347. Morgan JL, Darling AE, Eisen JA. Metagenomic sequencing of an in vitro-simulated microbial community. *PLoS One*. 2010 Jan;5(4):e10209.

348. Kircher M, Kelso J. High-throughput DNA sequencing--concepts and limitations. *Bioessays.* 2010 Jun;32(6):524–36.

349. Gilbert J, Meyer F, Bailey MJ. The Future of microbial metagenomics (or is ignorance bliss?). *ISME J.* 2011;5:777–9.

350. Hugenholtz P, Goebel BM, Pace NR. Impact of Culture-Independent Studies on the Emerging Phylogenetic View of Bacterial Diversity. *J Bacteriol.* 1998;180(18):4765–74.

351. Ringnér M. What is principal component analysis? *Nat Biotechnol*. 2008 Mar;26(3):303–4.

352. Malik R. Use of audit tools to evaluate the efficacy of cleaning systems in hospitals. *Am J Infect Control*. 2003 May;31(3):181–7.

353. Van Houdt R, Mijnendonckx K, Leys N. Microbial contamination monitoring and control during human space missions. *Planet Space Sci*. 2012 Jan;60(1):115–20.

354. Dancer S. How do we assess hospital cleaning? A proposal for microbiological standards for surface hygiene in hospitals. *J Hosp Infect.* 2004 Jan;56(1):10–5.

355. Williams AP, Avery LM, Killham K, Jones DL. Persistence of *Escherichia coli* O157 on farm surfaces under different environmental conditions. *J Appl Microbiol.* 2005 Jan;98(5):1075–83.

356. Noyce JO, Michels H, Keevil CW. Potential use of copper surfaces to reduce survival of epidemic meticillin-resistant *Staphylococcus aureus* in the healthcare environment. *J Hosp Infect.* 2006 Jul;63(3):289–97.

357. WHO. Infection prevention and control of epidemic-and pandemic-prone acute respiratory diseases in health care. 2007.

358. HPA. Healthcare-Associated Infection and Antimicrobial Resistance : 2010 / 11. 2010.

359. Brooke J., Annand J., Hammer A, Dembkowski K, Shulman S. Investigation of bacterial pathogens on 70 frequently used environmental surfaces in a large urban U.S. university. *J Environ Health*. 2009;71:17–22.

360. Lee L, Tin S, Kelley ST. Culture-independent analysis of bacterial diversity in a child-care facility. *BMC Microbiol*. 2007;7(27).

361. Andersson AM, Weiss N, Rainey F, Salkinoja-Salonen MS. Dust-borne bacteria in animal sheds, schools and children's day care centres. *J Appl Microbiol.* 1999 Apr;86(4):622–34.

362. Vos P, Garrity G, Jones D, Krieg N, Ludwig W, Rainey F, *et al*. Bergey's Manual of Systematic Bacteriology. Volume 3: The Firmicutes. 2nd ed. Williams & Wilkins; 2009.

363. Bokulich NA, Mills DA, Underwood MA. Surface Microbes in the Neonatal Intensive Care Unit: Changes with Routiene Cleaning and over Time. *J Clin Microbiol.* 2013;51(8):2617–24.

364. Hewitt KM, Mannino F, Gonzales A, Chase J, Caporaso JG, Knight R, *et al*. Bacterial Diversity in Two Neonatal Intensive Care Units (NICUs). *PLoS One.* 2013;8(1):e54703.

365. Oberauner L, Zachow C, Lackner S, Hogenauer C, Smolle K-H, Berg G. The ignored diversity: complex bacterial communities in intensive care units revealed by 16S pyrosequencing. *Sci Rep.* 2013;3:12.

366. Wexler HM. *Bacteroides*: the good, the bad, and the nitty-gritty. *Clin Microbiol Rev.* 2007 Oct;20(4):593–621.

367. Mezzatesta M., Gona F, Stefani S. *Enterobacter cloacae* complex: clinical impact and emerging antibiotic resistance. *Future Microbiol*. 2012;7:887–902.

368. Renvoise A, Harle J., Raoult D, Roux V. *Gordonia sputi* Bacteremia. *Emerg Infect Dis.* 2009;15(9):1535–7.

369. Mudshingkar S, Choure A, Palewar M, Dohe V, Kagal A. *Ochrobactrum anthropi* : An unusual pathogen: Are we missing them? *Indian J Med Microbiol.* 2013;31(3):306–8.

370. Kumar S, Kapoor S, Chadha S, Saigal S. *Ochrobactrum anthropi* septicemia and pneumonia in a preterm, small for gestational age infant with multiple congenital anomalies. *Indian J Pathol Microbiol.* 2013;56(3):317–8.

371. Chiu LQ, Wang W. A case of unusual Gram-negative bacilli septic arthritis in an immunocompetent patient. *Singapore Med J.* 2013 Aug;54(8):e164–e168.

372. Mages IS, Frodl R, Bernard KA, Funke G. Identities of *Arthrobacter* spp. and *Arthrobacter*-like bacteria encountered in human clinical specimens. *J Clin Microbiol.* 2008 Sep;46(9):2980–6.

373. Lemaitre F, Stein A, Raoult D, Drancourt M. Pseudoclavibacter-like subcutaneous infection: a case report. *J Med Case Rep.* 2011 Jan;5:468.

374. Kalyuzhnaya MG, De Marco P, Bowerman S, Pacheco CC, Lara JC, Lidstrom ME, *et al. Methyloversatilis universalis* gen. nov., sp. nov., a novel taxon within the Betaproteobacteria represented by three methylotrophic isolates. *Int J Syst Evol Microbiol.* 2006 Nov;56(Pt 11):2517–22.

375. Chou Y-J, Sheu S-Y, Sheu D-S, Wang J-T, Chen W-M. *Schlegelella aquatica* sp. nov., a novel thermophilic bacterium isolated from a hot spring. *Int J Syst Evol Microbiol.* 2006 Dec;56(Pt 12):2793–7.

376. Spring S. *Ottowia thiooxydans* gen. nov., sp. nov., a novel facultatively anaerobic, N_2O-producing bacterium isolated from activated sludge, and transfer of *Aquaspirillum gracile* to *Hylemonella gracilis* gen. nov., comb. nov. *Int J Syst Evol Microbiol.* 2004 Jan 1;54(1):99–106.

377. Yoon J-H, Kang KH, Oh T-K, Park Y-H. *Erythrobacter aquimaris* sp. nov., isolated from sea water of a tidal flat of the Yellow Sea in Korea. *Int J Syst Evol Microbiol.* 2004 Nov;54(Pt 6):1981–5.

378. Narciso-da-Rocha C, Vaz-Moreira I, Manaia C. Genotypic diversity and antibiotic resistance in Sphingomonadaceae isolated from hospital tap water. *Sci Total Environ*. 2014;1:466 – 467.

379. Poza M, Gayoso C, Gómez MJ, Rumbo-Feal S, Tomás M, Aranda J, *et al.* Exploring bacterial diversity in hospital environments by GS-FLX Titanium pyrosequencing. *PLoS One.* 2012 Jan;7(8):e44105.

380. Caporaso JG, Lauber CL, Costello EK, Berg-Lyons D, Gonzalez A, Stombaugh J, *et al.* Moving pictures of the human microbiome. *Genome Biol*. 2011 Jan;12(5):R50.

381. Fierer N, Leff JW, Adams BJ, Nielsen UN, Thomas S, Lauber CL, *et al*. Cross-biome metagenomic analyses of soil microbial communities and their functional attributes. *Proc Natl Acad Sci USA*. 2012;109(52):2–7.

382. Miroshnichenko M., Bonch-Osmolovskaya E. Recent developments in the thermophilic microbiology of deep-sea hydrothermal vents. *Extremophiles.* 2006;10(2):85–96.

383. Steven B, Leveille R, Pollard W., Whyte L. Microbial ecology and biodiversity in permafrost. *Extremophiles.* 2006;10(4):259–67.

384. Nightingale F. Notes on Nursing: What it is and what it is not. New York: D. Appleton and Company; 1859.

385. Hobday RA, Dancer SJ. Roles of sunlight and natural ventilation for controlling infection: historical and current perspectives. *J Hosp Infect.* 2013 Aug;84(4):271–82.

386. Kelley ST, Gilbert JA. Studying the microbiology of the indoor environment. *Genome Biol.* 2013 Mar 28;14(2):202.

387. Zarraonaindia I, Smith D, Gilbert J. Beyond the genome: community-level analysis of the microbial world. *Biol Philos*. 2013;28:261–82.

388. Rehman A, Lepage P, Nolte A, Hellmig S, Schreiber S, Ott SJ. Transcriptional activity of the dominant gut mucosal microbiota in chronic inflammatory bowel disease patients. *J Med Microbiol.* 2010 Sep;59(Pt 9):1114–22.

389. Schouls LM, Schot CS, Jacobs JA. Horizontal Transfer of Segments of the 16S rRNA Genes between Species of the *Streptococcus anginosus* Group. *J Bacteriol.* 2003;185(24).

390. Kitahara K, Miyazaki K. Natural and experimental evidence for horizontal gene transfer of 16S rRNA Revisiting bacterial phylogeny. *Mob Genet Elements.* 2013;e24210(February):1–6.

391. Delmont TO, Prestat E, Keegan KP, Faubladier M, Robe P, Clark IM, *et al.* Structure, fluctuation and magnitude of a natural grassland soil metagenome. *ISME J.* 2012 Sep;6(9):1677–87.

392. Schloss PD, Gevers D, Westcott SL. Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. *PLoS One.* 2011 Jan;6(12):e27310.

List of abbreviations

A	Adenine
ABI	Applied Biosystems
AIDS	Acquired immunodeficiency syndrome
ATCC	American Type Culture Collection
AV	Adenovirus
BHI	Brain-heart infusion
BSA	Bovine serum albumin
BSI	Bloodstream infection
C	Cytosine
CF	Cystic fibrosis
CFU	Colony-forming units
Cm	Centimetre
CMV	Cytomegalovirus
CNS	Coagulase-negative staphylococci
COSHH	Control of Substances Hazardous to Health
CTAB	hexadecyltrimethylammonium bromide
D&V	Diarrhoea and vomiting
DH	Department of Health
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
dsDNA	Double-strand DNA
EDTA	Ethylenediaminetetraacetic acid
EMP	Earth Microbiome Project
ESBL	Extended-spectrum beta-lactamase
G	Guanine
GI	Gastrointestinal
GOSH	Great Ormond Street Hospital
GRE	Glycopeptide-resistant enterococci
H_2O_2	Hydrogen peroxide
HCAI	Healthcare-associated infection
HCL	Hydrochloric acid
HMP	Human Microbiome Project
HPA	Health Protection Agency
IPC	Internal positive control
ITU	Intensive care unit
L	Litre
M	Molar

m	Metre
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MS	Mass spectrometry
MDE	multi-drug resistant Enterobacteriaceae
$MgCl_2$	Magnesium chloride
min	Minute
µl	Microliter
mm	Millimetre
mM	Milimolar
MMR	Measles, mumps and rubella
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSC	Microbiological safety cabinet
NGS	Next-generation sequencing
NHS	National Health Service
nM	nanomolar / nanomoles
nm	Nanometre
NV	Norovirus
^{O}C	Degrees, Celcius
PBS	Phosphate buffered saline
PC	Polycarbonate
PDV	Phocine distemper virus
PEG	Polyethylene glycol
pmol	Picomole
PTFE	Polyfluoroethylene
PTSD	Post-traumatic stress disorder
PV	Poliovirus
QIIME	Quantitative Insights into Microbial Ecology
qPCR	Quantitative PCR
rDNA	Ribosomal DNA
RH	Relative humidity
RNA	Ribonucleic acid
rpm	revolutions per minute
rRNA	Ribosomal RNA
RSV	Respiratory syncytial virus
RTI	Respiratory tract infection
RV	Rotavirus
SARS	Severe acute respiratory syndrome
SARS-CoV	SARS-coronavirus
SOP	Standard Operating Procedure
sp.	Species

spp.	species (plural)
ssRNA	Single-stranded RNA
T	Thymine
TB	Tuberculosis
TBE	Tris-Borate-EDTA
TSA	Tryptic soy agar
TVC	Total viable count
U	Units
UK	United Kingdom
UNG	Uracil-N-glycosylase
US	United States
UTI	Urinary tract infection
UV	Ultra-violet
VBNC	Viable but not culturable
VRE	Vancomycin-resistant enterococci
VZV	Varicella-Zoster virus
WHO	World Health Organisation

Appendix 1

Great Ormond Street Hospital for Children NHS Foundation Trust

Policy for microbiological screening of environment

LEAD DIRECTOR: Liz Morgan, supported by John Hartley,

POLICY APPROVED BY: John Hartley

DATE POLICY APPROVED: March 2013

IMPLEMENTATION DATE: March 2013

REVIEW DATE: March 2014

Introduction

A range of Trust policies are designed to reduce the risk of patients acquiring potentially pathogenic microorganisms by cross transmission from other patients, staff or visitors, or directly from the environment. Standard infection prevention and control precautions and environmental cleaning are adequate to reduce risk in most situations.

Policies are present as clinical guidelines:
http://www.gosh.nhs.uk/clinical_information/clinical_guidelines?category=I

However, some situations arise where additional controls are implemented for which microbiological monitoring is required to validate the control has been successfully applied.

Aims and objectives

The aim of this policy is to help reduce the risk of patients or staff acquiring potentially pathogenic microorganisms from the environment.

When microbiological screening of the environment is required

Environmental clearance for specific organisms:

Where a specific microorganism or clinical infections is recognised to lead to greater risk through environmental contamination, whether or not a cross transmission has occurred assurance is required that adequate cleaning has taken place beyond that demonstrated by the satisfactory standard checks (e.g. visual) after appropriate cleaning has been completed and before re-occupation of area.
Organisms and procedure listed below.

Confirmation of protective isolation ventilation:

Where increased patient susceptibility is present due to a severe immunodeficiency (congenital or acquired) and local risk assessment has designated requirement for a protective environment including HEPA filtered air. (Procedure below - Appendix 5: Environmental air screening protocol for critical ventilation systems supplying clinical areas**;** Schedule of sampling is in Estates Ventilation Policy).

Operating theatre environment – Validation of microbiological air standard is required at commissioning and annually as part of the planned preventative maintenance described in Estates Ventilation Policy (Testing procedure below)

Water quality – microbiological quality of water is monitored routinely for legionella and *Pseudomonas aeruginosa* (as described in Estates Legionella policy) and as required when investigations of specific organisms suggests water may be involved in the transmission.

How sampling is performed and the expected standard results are interpreted against

Procedures and standards are shown below in appendices

This policy does NOT cover

- Monitoring of air volumes and pressures as part of the validation of specialist ventilation units (schedule and parameters in Ventilation policy)
- Schedule for testing for legionella (see *Legionella* control policy) and *Pseudomonas aeruginosa* in water (to be included in Estates document in preparation).
- Microbiological monitoring of water in dialysis, decontamination department, hydrotherapy pool where separate policies exist
- Environmental monitoring in Pharmacy suites, gene or cell therapy, SSD, mortuary, laboratories or other research areas where separate polices exist.

Duties and responsibilities

Infection prevention and control (IPC) team – to update policy and undertake procedure. To teach other to undertake procedures as required by clinical areas and keep records.

Staff at ward level – to liaise with IPC, ensure area clean and ready; to undertake screening if trained

External contracted companies – to comply with these procedures

Procedure or guideline

Environmental clearance for specific organisms

Screening will only take place after appropriate adequate cleaning has been completed and signed off according to normal standards (Infection Clean Protocol GOS-EAF-PRO-10587)

Individual risk assessment is performed by the Infection Prevention and Control Team (IPC Team) to grade risk associated with any particular organism. The need to perform screening is dependent on the organism, any current outbreak/transmission and susceptibility of individuals who may be exposed.

Examples of organisms which may be screened for include:

Methicillin resistant *Staphylococcus aureus* (MRSA)
Multi-resistant gram negative species
Clostridium difficile
Vancomycin-resistant enterococci (VRE)

Norovirus
Adenovirus
Carbapenem-resistant Enterobacteriaceae and *Acinetobacter* species

Other organisms as determined by IPC Team

Preparation of bed spaces and patient areas

Bed spaces may be screened post-level 2 or 3 clean carried out by the cleaning contractor.

Bed spaces should be left for at least 2 hours post-clean to allow all surfaces to dry adequately.

Bed spaces need to have been checked by a cleaning supervisor and ward representative (as per cleaning SLA) before screening takes place and the bed space checking form must be signed to say that a check has taken place before screening is undertaken. Bed spaces must be visibly clean; if they are not then screening must be suspended until the room has been re-cleaned.

Bed spaces must be free of all disposable equipment, clinical equipment and linen. If this is not the case screening cannot be carried out until these are removed. Please note that the room is unlikely to be organism free if these items have not been removed pre-clean as they impede cleaning. It may be advisable to request the room be cleaned again before screening.

If the room has not been properly prepared or is still visibly dirty this must be flagged both to the cleaning contract supervisor and the senior ward staff member, as well as the IP&C team and the member of Facilities in charge of the cleaning contract.

If this situation is found an incident form must be completed.

Screening should be carried out by a trained member of staff. This may be a member of IP&C Team or a ward designated staff member.

After screening the bed space will be sealed until the results are back and a decision as to whether or not to open the room made.

Screening samples may be processed by a number of methods with availability of results differing between 1 and 5 days.

The decision to open the room must be made in conjunction with ward manager (who is expected to liaise with consultant staff) and IP&C and an individual risk assessment.

If a bed space is found to have two consecutive cleaning failures due to microbial detection after consultation with the IP&C Team then a meeting must be called to include the contract manager, the Mitie supervisor and appropriate ward staff and an action plan developed. (Infection Cleaning Policy/document library)

General Ward / Clinic Areas

General ward areas may be screened in response to cross transmission or outbreaks. Ward areas will be screened by a member of the Infection Control Team and may be carried out in relation to a requested level 2/3 cleaning instead of the routine ward clean.

When is Screening Necessary?

The risk assessment considers the organisms, underlying condition of host, potentially susceptible individuals, current transmissions, prior experience with this organism and any clinical consequences of delay in bed opening. Screening may be requested for specific very high risk organisms (highly transmissible or highly resistant) in any situation, or other organisms when involved in outbreaks.

MRSA

Experience has shown certain strains and patient conditions lead to increased risk of cleaning failure (despite visually satisfactory inspection). For some children, the IP&C team are able to alert these children on PIMS as carrying highly transmissible strains. Screening will be carried out post discharge of patients with a highly transmissible strain of MRSA after a level 3 clean. Screening may also be necessary with other strains in an outbreak situation.

Screening results will take 72 hours (longer if processed over a weekend)

Criteria for bed space reopening - all sites free from MRSA

Multi-resistant Gram negative species

A large number of children are present in the trust colonised with multi-resistant bacteria and routine trust screening, isolation and cleaning protocols are adequate to control most of these.

However, certain highly resistant bacteria may be present for which screening should be carried out post discharge. The alert that this is a highly resistant strain of gram negative microorganism should be included on the alert in PIMS. A level 3 clean is usually required pre-screen.

Screening results will take 72 hours (longer if processed over a weekend)

Examples include Enterobacteriaceae (*Klebsiella*, *Enterobacter* and *Escherichia*) or *Pseudomonas aeruginosa* carrying genes for transmissible carbapenemases, or multi-resistant *Acinetobacter baumanii* (MRAB), which have caused major outbreaks in other London hospitals.

Criteria for bed space reopening - all sites free from Multi-resistant Gram negative organisms

Clostridium difficile

Screening may be requested in response to outbreaks or in relation to specific patient factors.

C. difficile cases are usually sporadic in this trust, but the organism produces very resistant spores and outbreaks frequently occur in many health care settings. Screening will not be carried our routinely following detection of a case, but may be carried out in response to a request from the Infection Control Team/Consultant Microbiologist. Screening results will take up to 7 days

Criteria for bed space reopening - all sites free from *C. difficile*

Vancomycin-resistant *Enterococcus faecium* or *Enterococcus faecalis* (VRE)

Screening may be requested in response to outbreaks or in relation to specific patient factors.

Detection of VRE is uncommon in GOSH, but the faecally carried organism has been responsible for outbreaks in the past, usually felt to be propagated through environmental contamination despite cleaning. Screening will be considered post discharge of any patient colonised or infected with VRE, this should be included on the alert in PIMS and would require a level 3 clean pre-screen.

Screening results will take 72 hours (longer if processed over a weekend)

Criteria for bed space reopening - all sites free from VRE

Norovirus

Screening will be carried out in response to a request from the Infection Control Team / Consultant Microbiologist. Screening may also be requested in response to outbreaks and in rooms where the next admitted patient is likely to be immunosuppressed (e.g. Lion, Giraffe, Elephant and wards)

Screening results will take up to 72 hours (longer if processed over a weekend)

Criteria for bed space reopening - all sites free from Norovirus

Adenovirus

All cubicles occupied by Adenovirus excreting children which will next have severely immuncompromised children in (Fox, Robin and Butterfly BMT cubicles) must be screened after the level 3 clean post-discharge.

Screening may also be requested in response to outbreaks and in rooms where the next admitted patient is likely to be immunosuppressed (e.g. Lion, Giraffe, Elephant and wards)

Screening results will take up to 72 hours (longer if processed over a weekend)

Criteria for re-opening: Decision to open room before results will be assessed with respect to the risk in next room occupant.

Criteria for bed space re-opening:

The cubicle is opened with no further cleaning required if no site has an Adenovirus positive CT result of lower than 39.

If the cubicle has 2 sites positive with Adenovirus at CT's of no lower than 34, then those sites are re-cleaned twice using chlorine and the cubicle can be re-opened, as long as the sites positive to do include the floor inside the room. If the floor inside the cubicle is positive than the cubicle undergoes a repeat 'deep clean' and is re-screened.

If the cubicle has more than 2 sites with an Adenovirus positive CT of 34 – 38 or if any one site has a CT of lower than 34, then the entire room must have a repeat 'deep clean' and be re-screened in full before opening.

If the same objects fails to become negative after 3 cleans and screens, that object if possible is replaced within the cubicle and disposed of appropriately.

Step by step guide and flowchart

How to Carry Out Environmental clearance for Specific organisms

A set of predefined areas should be screened according to the attached protocols.

See appendix 1 – bacterial screening protocol
See appendix 2 – bacterial screening form
See appendix 3 – viral screening protocol
See appendix 4 – viral screening form

Ward and Other Environmental Screening

Ward Screening

General ward areas may be screened in response to cross transmission or outbreaks. Ward areas will be screened by a member of the Infection Control Team and may be carried out in relation to a requested level 2/3 cleaning instead of the routine ward clean. Screening results will take up to 7 days depending on the organism screened for.

Swab No.	Site	Area
1	Corridor floor outside of cubicle/ante room entrance of a known positive	10cm^2
2	Corridor floor outside of cubicle/ante room entrance of a known negative	10cm^2
3	Storage trolley surface outside of the room of known positive	10cm^2
4	Storage trolley surface outside of the room of known negative	10cm^2
5	Sluice work surface	10cm^2
6	Sluice room door handle (exit to corridor)	Entire handle
7	Macerator lid	Round the entire seal
8	Nappy weighing scales	Entire top surface 10cm^2
9	PC keyboards	Every key and surface on the right(~10cm^2)
10	Nurses station	10cm^2
11	Nurses station phone	Entire Keypad and handle
12	Notes trolley	10cm^2

Monitoring of areas with critical mechanical ventilation systems

Screening will be carried out by the IPC team, or a company that fulfils the screening criteria under direction of estates, under the following conditions:

- During commissioning
- As part of the annual maintenance schedule annual verification
- In areas where work has been undertaken on mechanical ventilation units providing protective HEPA filtered environment or operating theatre standard air quality where air quality may be altered (work on filter or down stream of filter)
- In areas supplied by mechanical ventilation where the fabric of the room has been breached.

All maintenance work or renovation in the clinical areas must be discussed with the Infection Control Team prior to implementation. Screening will be undertaken using the protocol set out in appendix 5 and form appendix 6.

Screening will involve the use of settle plates (blood agar alone is adequate) and air sampling (carried out prior to the placement of settle plates) onto blood agar. Sampling will be undertaken post level 3 clean and the room will be closed to entry throughout the sampling time. Screening time will depend on the area to be screened, but will take a minimum of three hours.
Screening results will take 72 hours (longer if processed over a weekend)

Plates are incubated according to laboratory protocol BSOP0058

Specifications for microbiological air sampling (BSOP0058)

Test types: **1 cubic metre air tests** (also called active air test or air test): collected using a calibrated air sampling device, 1 cubic metre of air

will be sampled on to a suitable agar plate. In house we use blood agar, although other non-selective agar is suitable.

Incubation: 18-24 hours at 35 -37^{O}C with initial 1 day cfu report.

Reading: Plates are read for colony count (fungi are identified to genus level)

Report: Bacterial count in cfu/m^3
Fungal count in cfu/m^3 (ensure report no Fungi grown and any growth is identified to genus level)

Settle plates: 9 cm blood agar plates left for 2 hours.

Incubation: 18-24 hours at 35 -37 ^{O}C with initial 1 day cfu report. Then 2 additional days at room temperature to give final report.

Reading: Plates are read for colony count (fungi are identified to genus level)

Report: Bacterial count in cfu/ plate
Fungal count in cfu/plate (ensure report no Fungi grown and any growth is identified to genus level)

Criteria for satisfactory validation – depends on the standard the area has been built and designed to e.g. HTM2025 or HTM 03-01

Theatres:
HTM 03-01 modified standards:
Non-HEPA filtrated area -10 or less cfu/m^3 total count
HEPA filtrated area -10 or less cfu/m^3 with no fungal colonies

HTM 2025 modified standards:
Non-HEPA filtrated area -35 or less cfu/m^3 total count
HEPA filtrated area -35 or less cfu/m^3 with no fungal colonies

Requirement for each area should be described in the Estates produced schedule but in summary:

Theatres:
Theatres are currently operating to 2025 (to which they were designed) but we aim to achieve 03-01 where possible, so theatre results that do not meet 03-01 total count need to be reviewed.

Protective isolation rooms and ward areas:
Newer PPVL rooms should meet HTM 03-01, with no Fungi detected

Older rooms and wards were built with HTM2025 standards.
If 03-01 standard is not meet the area needs review and individual decision made considering commissioning and annual verification records of what was achieved.

Areas requiring specialised ventilation screening:

- Operating theatres
- Clinical areas with HEPA filtration – Robin and Fox wards; Butterfly
- PICU, NICU, CICU, Angio Suite, HSDU
- All individual rooms providing protective isolation

Other critical ventilation systems, shown below, are **NOT** covered by this policy

- Pharmacy: Cytotoxic Suite, TPN Suite, Sterile Unit
- SSD
- Gene and cell therapy suites
  Areas should comply with good manufacturing clean room standards, administered by Pharmacy or research sponsors.
- Pathology laboratories. Covered by Pathology documents.
- Mortuary

Requirements for microbiological sampling

- Investigations will be undertaken by the Infection Control Team / Microbiology Department
- Any problems requiring urgent attention must be discussed with the Infection Control Team.
- Notification of the cleaning programme must be sent in advance, in writing, by the project leader concerned to the Infection Control Department
- Confirmation that the programme is on time, must be made by phone, by the project leader to the Infection Control Team (CNS or Infection Control Clinical Scientist) or Infection Control Doctor
- Appropriate arrangements for removal and storage of furniture, sterile and non-sterile stores must be made prior to commencement of any work.

Procedures to be undertaken prior to microbiological sampling

- Air flow and pressure parameters must be confirmed as meeting standards by Estates prior to any microbiological testing - Ventilation validation reports MUST be sent to Infection Control Team / Microbiology Department in time for these to be checked prior to sampling
- Level 3 clean (Infection Clean Protocol GOS-EAF-PRO-10587)
- Check that all appropriate windows and doors are closed
- Check that air conditioning is switched on. It must be ensured that both main and backup systems are fully functional.
- The area must be locked and left empty for a minimum of 2 hours prior to air sampling.
- It is the responsibility of the project leader concerned to inform the Infection Control Team that the area is ready for sampling.

Communication of results

- If testing is performed by an external company employed by Estates, result must be sent to Infection Control Team. Results will be made available by the Medical Microbiologist, or member of the Infection Control Team, who will phone and email the appropriate manager / project leader
- Where a microbiological failure is documented, individual advice on re-cleaning and sampling will be given by the Infection Control Team or Medical Microbiologist

Local arrangements for implementation

Who to Contact

Infection Control Team bleep 0640 ext 5284 and either the Virology or Microbiology lab as appropriate.

Who Should Carry Out Screening

This should be performed by a member of the Infection Control Team or a member of staff trained by the Infection Control Team; or company appointed by Estates

Distribution of Screening Results

The member of staff in charge of the ward will be contacted with the environmental screening results. If the cubicle/ward area is passed as clean the area is then available for use.

If the cubicle/ward area fails the first environmental screen the Infection Control team will discuss with the member of staff in charge what subsequent tasks need to be undertaken. The cleaning contract liaison and the cleaning supervisor will also be informed of any screening failure (as per the Infection Clean Protocol GOS-EAF-PRO-10587).

If repeat cleaning and screening is undertaken as a result of a failure the reporting process is the same. However if a cubicle/ward area fails its environmental screening more than twice a meeting will be called as detailed in the cleaning policy to evaluate why failures are occurring and how the situation can be resolved.

Archiving of screening results

Samples will be booked in to the laboratory computer system. Paper copies of results are not sent to clinical areas but will be archived in IP&CT office in a folder called Environmental Screening for future reference.

Training arrangements

Staff carrying out environmental screening should be trained and signed off as competent by a member of the Infection Control Team. A training update should be carried out annually and a training record maintained. Members of staff being trained to carry out environmental screening need to be trained to carry out both bacterial and viral sampling; as these procedures are different.

The training will be carried out by the IP&C team and record maintained locally by the ward or theatres.

Auditing and monitoring

Annual audit against Estates maintenance records to confirm microbiological air tested when appropriate

Annual audit against level 3 clean list to check screening was requested when appropriate

Annual audit training records for update

Audit that all external companies have followed our procedure

Appendices

Appendix 1: Screening Protocol

Bacterial Environmental Screening Method (MRSA and Multi-Resistant Gram Negative Organisms)

Introduction

Bacteria can be a source of contamination when a patient who is infected / colonised is present in a cubicle. These bacteria can also survive for long periods in the environment and so when a patient, with an alert requiring a level three clean, is discharged or moves room, we need to know that the cubicle is free from bacteria before placing another patient inside. The Infection Control team may ask that you sample (swab) a room after cleaning to make sure that all the bacteria have been removed.

What you need

Supplies needed on ward

- Permanent marker pen (to write on tubes)
- Charcoal swabs (kept in a clean dry location)
- Gloves
- Disposable aprons (white)
- Polythene specimen bag to put your samples in once taken
- Sterile water (from the clean utility)

Supplies provided upon request by bacteriology (phone bacteriology each time):

- **Bacteria Environmental Screening Form** (1 per cubicle - to be completed during screening by the person swabbing)

What you do

When you have been asked to screen a cubicle by the Infection Control Team:

Call bacteriology and ask for the number **Bacterial Environmental Screening Forms** (1/ cubicle). Tell them where you are screening and for which organism / alert.
Find out from the person in charge the information you need to fill out the **Bacterial Environmental Screening Form** and complete it.
When you have everything from the **what you need section** including gloves and aprons (taken from the clean utility room) put everything in a clean suitable place outside of the cubicle i.e. a fold table, or use a clean plastic tray which you can take into the room.
Wash your hands thoroughly with soap and water.
Put on a disposable plastic apron and gloves.
Enter the cubicle
Write on the **Bacterial Environmental Screening Form** in the **Swab No.** column the number of the swab you are taking (i.e. 1 – 12) and write in the **Site Swabbed** column which area you are going to sample (use the sites to be included table on the back of the form). Label the swab you are going to use with the swab number, site, cubicle number, ward and date. Only label one swab at a time!
Once your swab is labelled and the form written take a clean swab from its pack, being careful not to touch the cotton end.
Lightly moisten the swab in the sterile water.
Swab the area (use the **Area to be Swabbed** description in the table on the back of the form to help you)
Put the cotton end of the swab back into the tube containing the charcoal transport media.
Put the tube in the clean sample bag.
Collect the plastic caps to be thrown away in a clinical waste bin when you have finished taking all the swabs.
Repeat steps 7 and 8 with each swab to be taken.
If at any point you think you may have got something on your gloves you **must** change them for a clean pair.
If the room does not appear clean or the appropriate items (curtains etc) have not been removed report this to the nurse in charge or contact the Infection Control Team.
The swab remains and all gloves and aprons **must** go in a clinical waste bin. If you have used a tray make sure it is thoroughly washed with soap and water.
Make sure that you wash your hands thoroughly with soap and water.
Fill in the form on the cubicle door to say that sampling has been carried out.
Send the completed form and samples to bacteriology using the chute system.
Tell the person in charge that the screening has been completed.

Swab No.	Site	Area to be Swabbed
1	Floor under sink	4 $inch^2$/10cm^2
2	Bathroom door handle	Entire handle
3	Chair with arms	Both arms 4 $inch^2$/10cm^2
4	Oxygen outlet (above bed)	Entire surface
5	Telephone keypads	Entire keypad
6	Taps in Patient Bathroom	Entirety of both taps
7	Mattress top	4 $inch^2$/10cm^2
8	Bed/Cot frame under bed	4 $inch^2$/10cm^2
9	Trolley surface (in ante room if present)	4 $inch^2$/10cm^2
10	Side window sill (right hand side)	4 $inch^2$/10cm^2
11	Cubicle room exit door handle (cubicle side)	Entire handle
12	Corridor floor outside of cubicle/ante room entrance	4 $inch^2$/10cm^2

Additional sites should be swabbed (samples 13+) if there are any areas that look dusty or unclean. This should be reported back to the Infection Control Team.

Contact Numbers
Infection Control: 5284
Bacteriology lab 5280/8661
Consultant Microbiologist: 7930/5237/8594

Appendix 2: Screening Form

Bacterial Environmental Screening Form

Cubicle Tested: _____ **Ward**: _____

Patients ID: _____ **Patient discharge date**: _____

Date of Cleaning: _____ **Level of Cleaning**: _____

Bacteria: _____

Date: **Time**:

Laboratory No.	Swab No.	Site Swabbed	Culture Results
	1	Floor under sink	
	2	Bathroom door handle	
	3	Chair with arms	
	4	Oxygen outlet (above bed)	
	5	Telephone keypads	
	6	Taps in Patient Bathroom	
	7	Mattress top	
	8	Bed/Cot frame under bed	
	9	Trolley surface	
	10	Side window sill (right hand	

		side)	
	11	Cubicle room exit door handle (cubicle side)	
	12	Corridor floor outside of cubicle/ante room entrance	

Please inspect the room prior to screening. If the room is visibly dirty do not screen and inform the infection control team on ext. 5284/bleep 0640. Out of hours inform the PEC on duty. If any of the sites are not present select another site and list it on the form**. Always screen 12 sites.**

Name: _____ Signature:_____

Sites to be Included in Environmental Swabbing

Swab No.	Site	Area to be Swabbed
1	Floor under sink	10cm^2
2	Bathroom door handle	Entire handle
3	Chair with arms	Both arms ~5cm^2 on each
4	Oxygen outlet (above bed)	Entire surface
5	Telephone keypads	Entire keypad
6	Taps in Patient Bathroom	Entirety of both taps
7	Mattress top	4 inch^2/10cm^2
8	Bed/Cot frame under bed	4 inch^2/10cm^2
9	Trolley surface (in ante room if present)	10cm^2
10	Side window sill (right hand side)	4 inch^2/10cm^2
11	Cubicle room exit door handle (cubicle side)	Entire handle
12	Corridor floor outside of cubicle/ante room entrance	10cm^2

If any of the items on the above list are not there (i.e. mattress) or an anteroom is not present, please take at least 12 swabs. Choose which extra places/items to swab from the suggested list below. Please swab any areas which are visibly dirty and report them to the Infection Control Team. Make a note of where each extra sample was taken from on the **Bacterial Environmental Screening Form** in the space provided.

Suggested Additional Areas

Angle poise lamps
Television
Monitoring equipment
Bed lockers
Radiator grills

Contact Numbers
Infection Control: 5284
Bacteriology lab 5280/8661
Consultant Microbiologist: 7930/5237/8594

Appendix 3: Screening Protocol

Enteric Viruses Environmental Screening Method (Noro and Adeno Viruses)

Introduction

Enteric viruses can be a source of contamination when a patient who is infected is present in a cubicle. These viruses can also survive for long periods in the environment and so when a patient with adenovirus or norovirus is discharged or moves room, we need to know that the cubicle is free from virus before placing another patient inside. The Infection Control team may ask that you sample (swab) a room after cleaning to make sure that all the virus particles have been removed.

What you need

Supplies needed on ward (call virology when running low on swabs or pens):

- Permanent marker pen (to write on tubes)
- Sterile cotton wool swabs (kept in a clean dry location)
- Gloves
- Disposable aprons (white)
- Polythene specimen bag to put your samples in once taken

Supplies provided upon request by virology (phone virology each time):

- Batches of tubes of sterile water (12 per cubicle to be swabbed)
- **Enteric Virus Environmental Screening Form** (1 / cubicle - to be completed during screening by the person swabbing)

What you do

When you have been asked to screen a cubicle by the Infection Control team:

Call virology and ask for the number **Enteric Virus Environmental Screening Forms** (1 per cubicle) and tubes of sterile water (12 per cubicle) you need.
If out of hours call bacteriology for tubes - see contact numbers at the bottom of the sheet
Find out from the person in charge the information you need to fill out the **Enteric Virus Environmental Screening Form** and complete it.
When you have everything from the **what you need section** including gloves and aprons (taken from the clean utility room) put everything in a clean suitable place outside of the cubicle i.e. a fold table, or use a clean plastic tray which you can take into the room.
Wash your hands thoroughly with soap and water.

Put on a disposable plastic apron and gloves.
Write on the **Enteric Virus Environmental Screening Form** in the **Swab No.** column the
number of the swab you are taking (i.e. 1 – 12) and write in the **Site Swabbed** column which area you are going to sample (use the sites to be included table on the back of the form). Label the tube you are going to use with the swab number, label both the lid of the tube and the side of the tube. Only label one tube at a time!
Once your tube is labelled and the form written take a clean swab from the pack, being careful not to touch the cotton end.
Enter the cubicle to be screened, open the tube and lightly moisten the swab in the water.
Swab the area (use the **Area to be Swabbed** description in the table on the back of the form to help you)
Put the cotton end of the swab back into the water in the tube and break off the wooden end so that you can do up the lid.
Put the tube in the clean sample bag.
Collect the broken wooden ends to be thrown in a sharps bin when you have finished taking all your swabs.
Repeat steps 6 and 7 with each swab to be taken.
If at any point you think you may have got something on your gloves (i.e. water) you **must** change them for a clean pair.
Throw away the swab remains in the sharps bin and all gloves and aprons **must** go in a clinical waste bin. If you have used a tray make sure it is thoroughly washed with soap and water.
Make sure that you wash your hands thoroughly with soap and water.
Fill in the form on the cubicle door to say that sampling has been carried out.
Send the completed form and samples to virology using the chute system.
Tell the person in charge that the screening has been completed.

Swab No.	Site	Area to be Swabbed
1	Floor under sink	4 inch^2/10cm^2
2	Bedside Lamp controls	10cm^2
3	Chair with arms	Both arms 4 inch^2/10cm^2
4	Door handle into patient bathroom	Entire handle
5	Telephone keypads	Entire keypad
6	Taps in Patient Bathroom	Entirety of both taps
7	Mattress top	4 inch^2/10cm^2
8	Bed/Cot frame under bed	4 inch^2/10cm^2
9	Trolley surface (in ante room if present)	4 inch^2/10cm^2
10	Side window sill (right hand side)	4 inch^2/10cm^2
11	Cubicle room exit door handle (cubicle side)	Entire handle
12	Corridor floor outside of cubicle/ante room	4 inch^2/10cm^2

Contact Numbers

Infection Control: 5284
Virology Lab: 8507
Bacteriology lab (out of hours only): 5280/8661
Consultant Microbiologist: 7930/5237/8594

Appendix 4: Screening form

Enteric Viruses Environmental Screening Form (Noro/Adeno)

Cubicle Tested: _____ **Ward**: _____

Patients ID: _____ **Patient discharge date**: _____

Date of Cleaning: _____ **Level of Cleaning**: _____

Virus: _____

Date: **Time**:

Laboratory No.	Swab No.	Site Swabbed	Culture Results
	1	Floor under sink	
	2	Bedside Lamp controls	
	3	Chair with arms	
	4	Door handle into patient bathroom (cubicle side)	
	5	Telephone keypads	
	6	Taps in Patient Bathroom	
	7	Mattress top	
	8	Bed/Cot frame under bed	
	9	Trolley surface (in ante room if present)	
	10	Side window sill (right hand side)	
	11	Cubicle room exit door handle (cubicle side)	
	12	Corridor floor outside of cubicle/ante room entrance	

Please inspect the room prior to screening. If the room is visibly dirty do not screen and inform the infection control team on ext 5284/bleep 0640. Out of hours inform the PEC on duty. If any of the sites are not present select another site and list it on the form**. Always screen 12 sites.**

If the room is being screened for Adenovirus (BMT patients):

- Any pillows should have been thrown away prior to the room being cleaned. Please indicate if this was the case _____
- The clinical waste bin should have been sent to MEDU prior to the room being cleaned. Please indicate if this was the case _____

Name: _____ Signature: _____

Sites to be Included In Environmental Swabbing

Swab No.	Site	Area to be Swabbed
1	Floor under sink	10cm^2
2	Bedside Lamp controls	10cm^2
3	Chair with arms	Both arms ~5cm^2
4	Door handle into patient bathroom	Entire handle

5	Telephone keypads	
6	Taps in Patient Bathroom	Entirety of both taps
7	Mattress top	
8	Bed/Cot frame under bed	
9	Trolley surface (in ante room if present)	$10cm^2$
10	Side window sill (right hand side)	4 $inch^2$/$10cm^2$
11	Cubicle room exit door handle (cubicle side)	Entire handle
12	Corridor floor outside of cubicle/ante room	$10cm^2$

If any of the items on the above list are not there (i.e. mattress) or an anteroom is not present, please still take 12 swabs. Choose which extra places/items to swab from either the suggested list below, or areas which are visibly dirty. Make a note of where each extra sample was taken from on the **Enteric Viruses Environmental Screening Form** in the space provided.

Suggested Additional Areas

Angle poise lamps
Television
Monitoring equipment
Bed lockers

Contact Numbers

Infection Control: 5284
Virology Lab: 8507
Bacteriology lab (out of hours only): 5280/8661
Consultant Microbiologist: 7930/5237/8594

Appendix 5: Environmental air screening protocol for critical ventilation systems supplying clinical areas

Method for Environmental Air Screening of Critical Ventilated Clinical Areas

Introduction

Microbiological air sampling of mechanically ventilated clinical areas designated 'critical' is required at commissioning and regular validation verification, as stipulated in the Ventilation policy.
Each critically ventilated area will have a 'schedule' (currently under protection by Estates) describing the design and validation standards.
Non-HEPA filter supplied areas will usually be designed to 'Theatre standard' (</= 10 or less cfu/m^3 if commissioned against HTM 03, </= 35 cfus if HTM 20:25), while those areas with HEPA filtration (protective isolation rooms, ward common areas, or HEPA filtered theatres) will be expected to provided standard theatre air plus additionally be free of detectable fungus.

Test selection and number of samples:

Routine theatre standard air testing - Routine air testing will be carried out with a validated air sampling device. Currently the Sampl'air sampler is available to

Infection Control but other validated devices are suitable as long as they test a minimum of $1m^3$ of air.

One $1m^3$ air test will be performed per room e.g. operating theatre, preparation room, patient bedroom, room antechamber; multiple tests in large areas e.g. one per bed space in open HEPA filtered unit. The device will be located in centre of area where patient usually sited.

Additional fungal testing in HEPA filtered areas - Additional tests for bacterial and fungal growth will be performed by use of settle plates.

4 plates per operating theatre or room; two per smaller areas e.g. prep or antechamber.

Before testing ensure ventilation is to standard and area is clean
Do not test a commissioned or validated area unless Estates (or designated company) have confirmed the mechanical ventilation if performing to standard volume, flow and pressure regimen.

What you need:

Supplies required:

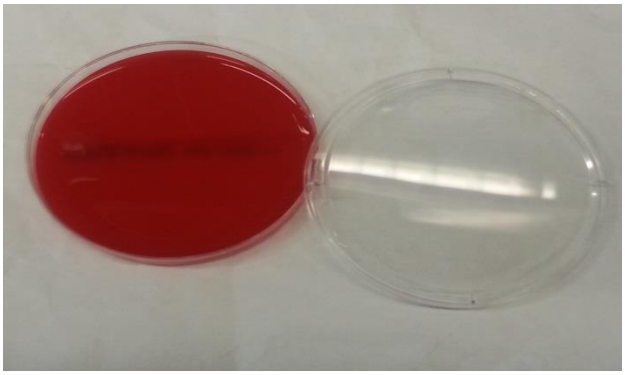
- Permanent marker pen (to write on agar plates)
- Gloves x 2
- Disposable overshoes x 2
- Disposable theatre cap x2
- Scrubs
- Surgical mask x 2
- Sterile/disposable gown x2
- Polythene specimen bag to put your samples in once taken
- Rubber bands (to band your plates together for safe transportation)
- Agar plates
- One blood agar plate for each air sample
- Four blood plates for each bedspace or theatre area sampled
- Two blood plates for each antechamber sampled
- Sterile filter heads for the air sampler
- Sampl'air Lite air sampler
- Tape (to tape up the room whilst settle plates are down)
- Notice (to say keep out sampling underway)
- **Environmental Screening of Mechanically Ventilated Rooms Form** (1 per area - to be completed during screening by the person carrying out the screening)
- Smoke testing equipment pack
- Tray/autoclave bag to hold equipment

What you do:

Charge Sampl'air lite overnight and ensure that filter heads have been autoclaved
Before agreeing to sample ventilation performance should be confirmed as working to specification with the measurement details sent to estates and to the IP&CT and

a level 3 clean has been carried out (wait at least 60 minutes after cleaning before sampling to let the chlorine dry thoroughly)
Before sampling inform the microbiology lab that there will be environmental samples collected for processing
Collect equipment into a tray or autoclave bag and change into scrubs
When you arrive at the room ensure there is a cleaning sign off sheet on the door indicating that a level 3 clean has been carried out
Wash hands with soap and water (where available), alcohol if not
Don overshoes, cap, gown, mask and gloves in that order
Visually inspect the room for cleanliness and for breaches in the building fabric. Ensure all clinical equipment and fabric items such as curtains and pillows have been removed. If room has not been cleaned or there is anything that would affect sampling **DO NOT SAMPLE!** Check with estates and IP&CT team
Undertake smoke testing of the room to ensure the direction of air flow is as expected. If not as expected **DO NOT SAMPLE!** Check with estates and IP&CT team
Break the smoke tube so that smoke is released being careful not to inhale
Attach the bulb and press to produce smoke along the door frames, plug sockets and other sources of ingress/egress of air
Label blood agar plates using a permanent pen as written on the **Environmental Screening of Mechanically Ventilated Rooms Form**
Include date and time of sampling, room sampled, air sample, initials of person sampling
Take a labelled blood agar plate and fit it to the Sampl'air lite
Remove the protective cover from the top of the air sampler
Remove the lid of the agar plate
Clip the base of the agar plate into place on the top of the air sampler
Place the lid of the agar plate onto the opened sterile filter pack (see step 12)
Attach the sterile filter head to the Sampl'air Lite:
Open the sterile pack without touching the inside of the pack
Lift the filter from the pack by its sides without touching the top filter section
Attach the filter head onto the top of the Sampl'air Lite, covering the attached agar plate without touching it
Press the on button on the Sampl'air Lite this will bring up a screen saying SAMPLE
Press the green arrow button; this brings up a message saying 10Min
Press the right arrow button which changes the 10Min message to 1000L
Press the green arrow button again, this bring up a message saying START 01:00 (indicating the sampler will start with a 1 minute delay)
Enter the first room to sample (always start in the room with the cleanest air and work your way backwards e.g. theatre prep, main theatre, anaesthetic room. Place the sampler in the centre of the room, if possible at waist height and press the green arrow button to start the sampler. You will then have 1 minute to vacate the space before the sampling starts. (You can also start the sampler with a remote from the doorway if you can get a good angle to the sampler)
You must remove yourself from the space that you are sampling completely and make sure that all doors are closed. When approaching the doorway to the sampling space you should be able to hear the sampler beeping if it has finished. If there is an observation window the sampler has a bar that fills and a counter that clocks up as sampling is completed
When the sampler has finished collect the sampler being sure not to touch the filter section on the top.

Remove the filter section by holding the sides, ensuring you do not touch the top section.
Place the filter clean side down on the sterile wrapping (e.g. with the top filter section raised above the paper) and place the lid of the agar plate onto the agar plate before removing it and placing in a specimen bag
Repeat this process for each room to be sampled. Change filter heads if you ever suspect a contamination event might have occurred or after every 10 air samples taken
When all rooms have had an air sample taken label up settle plates for each room. Settle plates should be labelled as per the Environmental Screening of Mechanically Ventilated Rooms Form
Labelling should include date and time of sampling, room sampled, position within the room and initials of person sampling.
Plates should be positioned with the lid of the plate balanced against the edge of the agar plate itself (see picture below).



In a room with antechamber, settle plates should be placed in the room farthest from exit first, being careful not to contaminate them via touch or aerosolized droplets. Plates should then be placed in the antechamber before exit. In a theatre plats should be placed in prep room, then theatre.
When settle plates have been placed leave the sampling area and tape up the entrance to prevent others accessing the sampling area, put up notice saying Infection Control Sampling Underway. Use an elastic band to collect together air sampling plates and remove PPE
Give air sampling plates and form to bacteriology for processing (plates will be incubated at 37^{o}C for 48 hours and will be read for total viable counts at 24 and 48 hours)
Settle plates must be left for a minimum of two hours before collection. When collecting settle plates appropriate PPE must again be worn, remove tape and signage. Sign and date the Mitie form to say sampling has been undertaken.
Give settle plates to bacteriology for processing (plates will be incubated at 37^{o}C for 24 and room temperature for a further 48 hours. Plates will be read for total viable counts and fungal growth at 24, 48 and 72 hours.
Email the appropriate project team to say that sampling has been undertaken and with a date that the expected results are due.
When microbiology results are available phone the projects team to let them know whether the room has passed or failed and confirm by email (sent to the IP&CT, projects team and estates and facilities)

Appendix 6: Screening Form

Environmental Screening of critical ventilated clinical areas: Form

Area Tested: _____ **Date of Cleaning**: _____

Level of Cleaning: _____ **Smoke testing as expected (circle)**: ☒ ☑

Reason for screening:

_____

Laboratory No.	Sample Type (air or settle plate)	Site	Culture Results

Please inspect the room prior to screening. If the room is visibly dirty do not screen and inform the infection control team on ext. 5284/bleep 0640. Out of hours inform the floor manager.

Name: _____

Signature:_____